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REPLICATION OF SUBSTRATE DETAIL BY BARNACLES AND SOME OTHER MARINE ORGANISMS

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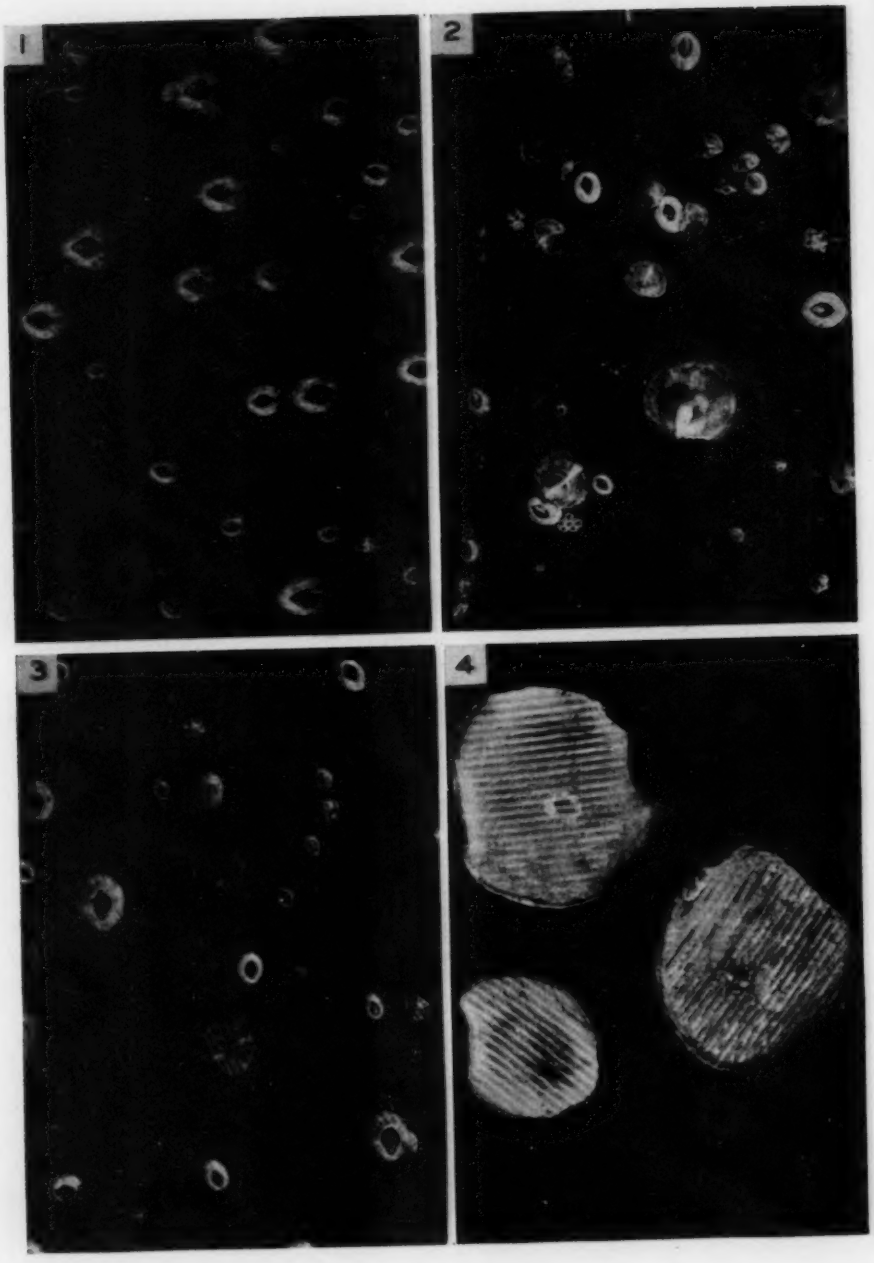
Many marine organisms exist for a short time as free swimming larvae before attaching themselves to a suitable surface and entering a new phase of their life cycle as sedentary individuals. Among these organisms are the acorn barnacles and some species of molluscs. Pomerat and Weiss (1946) found that glass plates with various surface textures to which the cypris larvae of the barnacles *Balanus amphitrite niveus* Darwin and *Balanus improvisus* Darwin attach does not affect the frequency of successful attachments. However, the writer found that the nature of the surface affects the orientation of the long and short axes of *Balanus eburneus* Gould and the topography of the calcareous compartments of *Balanus eburneus* Gould and the shells of the mollusc *Ostrea virginica* Gmel. Furthermore it is evident that the basal plate of the barnacle is subject to variations in form and topography according to the surface upon which growth takes place.

In order to study orientation phenomena and variations in form, it was necessary to use a surface of attachment affording consistent irregularities and for this purpose ordinary phonograph records were well adapted.

The records were submerged in horizontal and vertical positions several feet beneath the lowest tide level in the Mississippi Sound at Bayou La Batre, Alabama, and at the U. S. Fish and Wildlife Service laboratory on Santa Rosa Sound at Pensacola, Florida. Barnacle and oyster larvae of the species mentioned were found to attach readily and in large numbers to the black surfaces of the records. The age and size of the organisms ultimately collected depended, of course, upon the length of time the records were allowed to remain in the sea. The earliest examination of the records was made following a fourteen-day period of submergence. Many of these barnacles were assumed to be twelve to fourteen days old with regard to sedentary life, as it is possible for very large numbers of barnacle larvae to become attached during the first forty-eight hours of the submergence of a surface (Pomerat and Reiner, 1942).

The grooved surface offered by the phonograph records influenced the orientation of the barnacles to the extent that on one record 443 barnacles of a sample population of 663 had grown with their long axes parallel to the grooves in the record while the remaining 220 grew at various other degrees from parallel (Fig. 1). The 95 per cent confidence interval for this sample indicates that of the total number of barnacles attaching to the record, some 63 per cent to 71 per cent orient themselves

PLATE I



parallel to the grooves while not more than 37 per cent orient themselves at degrees other than parallel. The barnacles attached to the smooth surfaces of the records used as controls were oriented at random (Figs. 2 and 5). This fact tends to eliminate the possibility that geotropic or phototropic factors are greatly involved in the orientation of cypris larvae parallel with the grooves of the record. The oysters whether on the smooth or the grooved surface of a record did not show any tendency to become oriented and were attached at random (Figs. 2 and 3).

The external surfaces of the calcareous compartments of the barnacles were found to have duplicated the grooved pattern of the record while the basal plates which were in immediate contact with the record were found to have become ridged in a pattern similar to that of the record (Figs. 3, 4, 6, 7, 8). The grooves were of approximately the same width and depth and were continuous with those of the record. The grooves which appear on the compartments of the barnacle persist during subsequent growth although they were inclined to the right or left with respect to the grooves in the record with which they were continuous (Fig. 8). This shifting in space laterally occurs because of the movements of the individual compartments in the course of growth. The grooving of the record was not found to have affected the internal surfaces of the basal plate or the compartments of the barnacle. The terga and scuta were not affected in any way either externally or internally.

The ratio between length and width of *Balanus eburneus* Gould in many instances was affected by the presence of the grooves of the record and in consequence the shape of the barnacle was changed during growth. Regardless of the orientation the particular compartments and the parts of the basal plate of the barnacle that had to continue growth in a direction perpendicular to the grooves in the record were retarded in the rate of increase in length or width in the plane of attachment (Fig. 7).

This grooved and ridged condition was also found on the external and internal surfaces of both shells of the oyster, the grooved pattern of which was located on the external upper shell and the ridged pattern of which was located on the surface of the shell in contact with the record (Fig. 3). The grooves and ridges were well defined in the young oyster over the entire upper and lower shells both internally and externally, but they became somewhat obliterated after several months of age except at the extreme growing edges.

Other species of barnacles *Balanus improvisus* Darwin and *Balanus amphitrite niveus* Darwin were investigated by the use of phonograph records by Dr. C. M.

EXPLANATION OF THE FIGURES

A comparison of living barnacles and oysters growing on the grooved and smooth surfaces of phonograph records. All barnacles and oysters are of the species *Balanus eburneus* Gould and *Ostrea virginica* Gmel., respectively.

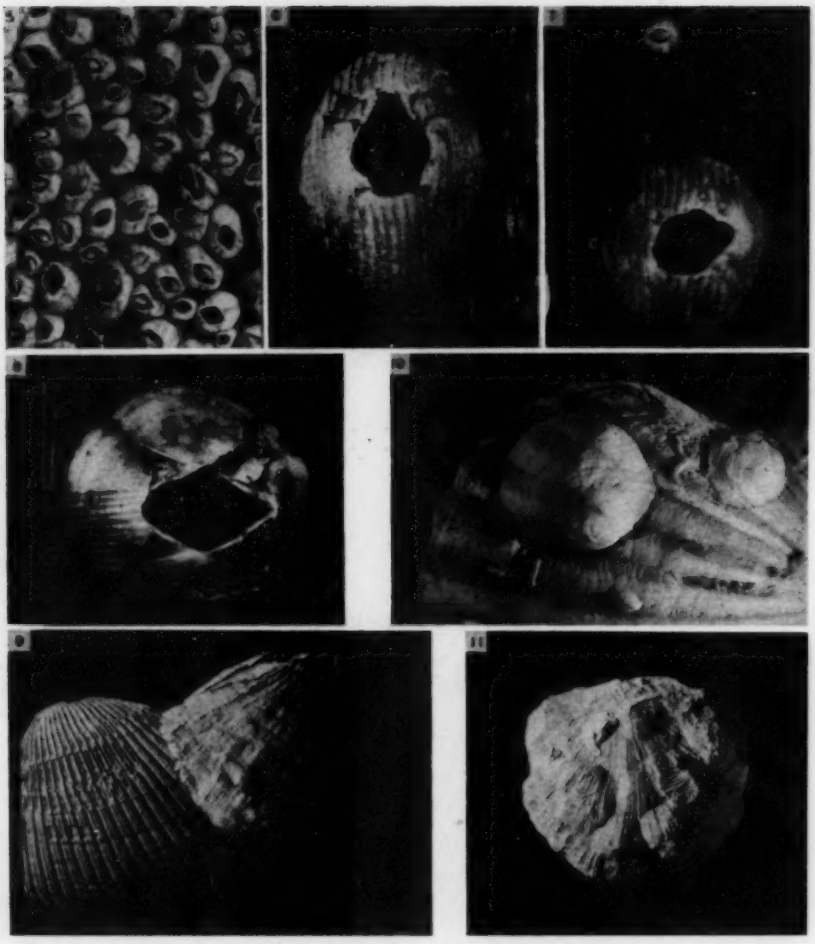
1. Orientation of the long axes of the barnacles parallel with the grooves of the phonograph record. (×4)

2. Random orientation of barnacles and oysters on the smooth surface of the record. Note that several barnacles are oriented along the groove at the left edge of the figure. (×4)

3. Random orientation of the oysters with the grooves of the record and showing grooves on the upper shell of the oyster (lower middle of figure) and grooves on the barnacles. (×4)

4. Several barnacles removed from the phonograph record showing the acquired ridging on their basal plates. (×4)

PLATE II



EXPLANATION OF THE FIGURES

A further comparison of living barnacles growing on phonograph records, a fossil barnacle, a living mollusc, and a fossil brachiopod attached to various substrates.

5. Random orientation of *Balanus eburneus* Gould growing on the smooth surface of a record. (× 4)

6. Grooving on the compartments of the barnacle *Balanus eburneus* Gould which are continuous with the grooves in the record. (× 12)

7. Two barnacles (*Balanus eburneus* Gould) growing with their long axes perpendicular to the grooves of the record showing a marked departure from the normal length-width ratio and showing grooving of the compartments. (× 12)

Pomerat (personal communication). These species, however, failed to show any such striation of the compartments. This would suggest a different pattern of calcium deposition in compartment formation.

Pilsbry (1916) found that the living barnacles, *Balanus rostratus apertus* Pilsbry and *Balanus balanus* Linnaeus, when growing on *Pecten* were capable of reproducing the ribs of the scallop's shell on its own compartments.

Darwin (1854) offered an explanation for the fact that the shape of sessile barnacles depended on their position and grouping while the surface of attachment has a great effect on that of the shell. As the walls are added to at their bases, every portion has at one time been in close contact with the supporting surface and, therefore, a strongly ribbed species such as *Balanus porcatus* DaCosta might closely resemble a nearly smooth species such as *Balanus crenatus* Bruguière. However, both were found to have a peculiar appearance owing to their attachment to a species of *Pecten*. Furthermore specimens of *Balanus patellaris* Spengler have been found which were curiously pitted like the wood to which they had adhered.

Among the fossil forms which exhibit similar phenomena is the barnacle *Balanus concavus* Bronn from the Miocene found attached to a ribbed mollusc from which it had obtained a ridged surface (Fig. 9). Pilsbry (1930) found that the compartments of the fossil barnacle *Balanus shilohensis* (Pilsbry) of the Miocene were smooth except when adventitiously sculptured by growth on a ribbed shell or other rough support.

A mollusc, *Anomia simplex* d'Orb, which was found growing on a ridged *Pecten* shell, exhibited the persistence of acquired ridging and grooving on its own shell (Fig. 10).

Hall (1870) found that the surface of the valve of the brachiopod *Crania scabiosa* (Hall) [*Petrocrania scabiosa* (Hall)] was sometimes obscured by the roughness of the substance to which it is attached and caused irregular growth by which it often assumed the features of the foreign body. Figure 11 shows the resulting irregularities of an Upper Ordovician *Petrocrania scabiosa* (Hall) when growing on the shell of *Rafinesquina ponderosa* Hall. The fossil brachiopod *Crania socialis* Hall [*Petrocrania scabiosa* (Hall)] was also reported by Hall (1892) to have maintained the marks of the sutures between the segments of a crinoid to which it was attached. Among the Pelecypods attachment by cementation has been found to result in a strongly vesicular structure in the attached shell. That this phenomenon may be a more general rule is emphasized by certain attached annelidan tubes such as *Cornulites proprius* Hall. Furthermore marked vesiculation of the shell has been found among brachiopods such as *Petrocrania* and *Richthofenia*, which are attached by a considerable surface of the shell (Hall, 1892).

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8. The barnacle, *Balanus eburneus* Gould growing both on the grooved and the smooth surface of the record. Note the angle of the grooves on the compartments with respect to the surface of the record. (× 8)

9. The fossil barnacle *Balanus concavus* Bronn attached to a *Pecten* showing grooving on its own compartments. (× 1.5)

10. The living mollusc *Anomia simplex* d'Orb attached to a *Pecten* showing grooving on its own shell. (× 2.5)

11. The fossil brachiopod *Crania scabiosa* (Hall) [*Petrocrania scabiosa* (Hall)] showing the acquired ridging derived from the shell of *Rafinesquina ponderosa* Hall. (× 1.5)

Kozlowski (1929) found that the fossil brachiopod *Philhedra mimetica* Kozlowski growing on the shell of *Camarotoechia* (*Wilsonia*) *tarda* (Barrande) reproduces the ridges. In addition the fossil brachiopod *Philhedra crenistriata* (Hall) was found capable of reproducing the substrate upon its own shell.

Discussion

The tendency for larvae of the barnacle *Balanus eburneus* Gould to become oriented with their long axes parallel with the grooved surface offered by the phonograph record was regarded as having occurred at the initial attachment when the cypris larvae were of an elliptical shape. Although the barnacles exercise slight movements in becoming firmly attached immediately following metamorphosis from the cyprid stage to the adult, the majority of them maintain their original position. It is quite possible that because of the elongate shape of the barnacle cyprid it more easily attaches itself parallel to the grooves than in other positions. The fact that oyster larvae are more or less radially symmetrical would seem to eliminate any possibility other than random chance of establishing their body axes in a particular direction when attaching to a phonograph record.

It is well known that the increase in length of the individual compartments of the barnacle is due to the secretion of building material from the lower edges of the valves (Darwin, 1854) and at the outer edges of the basal plate. This is substantiated by the fact that the grooves on the barnacle persist to the upper edges of the compartments. The secretion of the shell building material onto the grooved substrate leaves no other course for the building material than to arrange itself to conform with the pattern offered by the substrate. With subsequent secretions the building material is arranged similarly and eventually a continuous groove or ridge appears.

The oyster also became grooved and ridged while growing on phonograph records, as did an *Anomia simplex* d'Orb when attached to *Pecten* due to the fact that the mantle secreted the building material onto the available substrate and the material was obliged to follow the contours of the surface. In these two molluscs the lower shells reproduce the characteristics offered by the substrate and, therefore, the upper shells conform to the pattern conceived by the lower shells.

There are variations in the normal length-width ratio of many barnacles growing on a grooved surface. This may be attributed to the following fact. In order for the compartments and the parts of the basal plate of the barnacle growing perpendicular to the grooves in the record to attain the same linear proportions at the same rate as would its partner on a plain surface, a greater total surface area must be covered by the shell building material and in consequence a greater amount of time and material is required.

While all species of barnacles adhere closely to the substratum, not all barnacles reproduce the pattern of the substrate upon which they grow. It would seem that the species which do so may gain some selective advantage by the mechanical mimicking of a substrate pattern upon their compartments. This duplicating phenomenon is probably not merely a consequence of gaining an adequate fixation to the surface.

SUMMARY

1. Barnacles of the species *Balanus eburneus* Gould were found to orient themselves with their long axes parallel to the grooves in the substrate, while oysters (*Ostrea virginica* Gmel.) were found to orient themselves at random.

2. The grooving in the substrate was found to affect the topography of the compartments and the basal plate of the barnacle *Balanus eburneus* Gould. The grooves are duplicated upon the compartments and the ridges upon the basal plate. Any one groove on the record causes a ridge to occur on the basal plate and a groove to appear on the compartments of the barnacle. These grooves and ridges are initiated at the early growth stages and persist irrespective of the ultimate size of the organism.

3. In a similar manner the grooved surface of the phonograph records affects the shells of the mollusc *Ostrea virginica* Gmel. both externally and internally by causing grooves and ridges to appear on the upper and lower shells.

4. The living species of barnacles, *Balanus rostratus apertus* Pilsbry, *Balanus balanus Linnaeus* (Pilsbry, 1916), *Balanus porcatus* DaCosta, *Balanus crenatus* Bruguière, *Balanus patellaris* Spengler (Darwin, 1854), and the fossil barnacles *Balanus shilohensis* (Pilsbry) (Pilsbry, 1930) and *Balanus concavus* Bronn also have their compartments affected by the substrate to which they attach.

5. The mollusc *Anomia simplex* d'Orb may also exhibit characteristics of the surface to which it attaches.

6. The fossil brachiopods *Crania scabiosa* (Hall) [*Petrocrania scabiosa* (Hall)] (Hall, 1870), *Philhedra mimetica* Kozlowski (Kozlowski, 1929), *Philhedra crenistriata* (Hall) and the annelid tube of *Cornulites proprius* Hall (Hall, 1892) have been found to assume certain features of the substrate upon which they grew.

7. Grooving and ridging occur on the barnacle *Balanus eburneus* Gould because the shell building material secreted by the organism in maintaining close contact with the substrate is obliged to follow the contours of the surface. This close association during growth eventually leads to the formation of grooving on the compartments and ridging on the basal plate.

8. A similar explanation for the fact that young of the molluscs *Ostrea virginica* Gmel. and *Anomia simplex* d'Orb show ridging and grooving phenomena is offered, with the exception that the lower shell of a mollusc acts as the pattern to which the upper shell must conform during growth. This explanation is probably adequate for similar substrate reproduction by the brachiopods.

9. Variations in the normal length-width ratio of *Balanus eburneus* Gould result from growth upon a grooved surface. This was attributed to the fact that the compartments and the parts of the basal plate which must grow perpendicular to the grooves are retarded in the rate of increase in length or width in the plane of attachment.

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SPINDLE DEVELOPMENT AND BEHAVIOR IN THE GIANT AMOEBA [1]

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The anaphase movement of chromosomes has long been a problem, many details of which are still unsolved. The elongation of the interzonal region of the spindle as first postulated by Belar, seems to play an important part in forcing groups of daughter chromosomes apart. The mechanism by which this elongation is accomplished has not been made clear. Evidence bearing on the question was presented by Short (1945), who found 20 anaphase and telophase spindles twisted to the right in the giant amoeba, when the groups of daughter chromosomes were over 17 microns apart. None were twisted to the left. "The constancy in the direction of the twisting would result from a property inherent in the spindle apparatus; possibly an uncoiling of spindle elements as elongation proceeds." Since no previous observations of interzonal twisting, constant in direction, have been recorded, and the observations of Short are limited to 21 spindles in one amoeba, it is important to determine whether the direction of twisting is always the same in different individuals and whether this twisting is always associated with elongation. Such determination may add much to our understanding of the mechanism of elongation and of the nature of the interzonal fibers as well.

The giant amoeba is favorable material for a study of the mitotic spindle. It is easily cultured in the laboratory and multiplies rapidly. It possesses a large number of nuclei, all of which divide at essentially the same time. These dividing nuclei are not confined within a limited space. The majority of divisions allow for spindle elongation without obstruction by a nearby wall or cell membrane. The living amoeba has a somewhat characteristic appearance at different mitotic stages, making it possible to select desired stages for study. The work reported here has been confined to those stages of mitosis in which the spindle is apparent, and has been concerned primarily with the direction and constancy of interzonal twisting.

Throughout this paper the term *interzonal* will be used when referring to that part of the spindle which is seen between the separating groups of daughter chromosomes. This part may be made up of continuous fibers as well. The term chromosomal fibers has been applied to that part of the spindle extending from

[1] The proper name of this organism has been the subject of considerable controversy for a number of years. It is variously known as *Chaos chaos, Pelomyxa carolinensis,* and *Amoeba carolinensis.* Recent discussions of the validity of the various names have been presented by Short (1946), Kudo (1946), and Wilson (1947). Authorities for the taxonomic names are found in these papers.

[2] This work was done at the Miller School of Biology, University of Virginia, in the session 1945–46. It fulfills in part the requirements for the degree of Master of Science. The writer wishes to express his indebtedness and sincere appreciation to Dr. Ladley Husted for helpful advice, assistance and encouragement, and to Mr. R. B. Short, who made his slides available.

each group of daughter chromosomes toward the polar region. The direction of twisting of the interzonal spindle has been referred to as "right-hand" or "to the right." This is intended to mean that if one observes a *side-view* of an interzonal spindle (as seen from either end, not to be confused with a polar-view) fibers at the uppermost focal level are seen to cross from the observer's left side of the nearest chromosome plate, to the observer's right side of the more distant plate, while fibers at the lowermost focal level cross from the observer's right of the nearer plate to the observer's left of the distant plate.

Observations made during the course of this study show that "pushing" forces are involved in anaphase separation. The data obtained show that a right-hand twist of the interzonal spindle, accompanied by an apparent relaxation of the fibers as the separation of the groups of daughter chromosomes continues, consistently appears when these groups have moved 18 to 20 microns apart.

Material and Methods

The original stock culture was obtained from the General Biological Supply House, Chicago, Illinois, in December 1945. The amoebae were grown in butter dishes half-filled with wheat culture medium, containing paramecia, rotifers, bacterial gloea, numerous ciliates and water molds. No difficulty was experienced in maintaining cultures in vigorous condition at ordinary laboratory temperatures at any time of the year.

Specimens selected from stock cultures were fixed with Carnoy's fixative, and stained with either Delafield's or Heidenhain's haematoxylin. The amoebae were mounted in diaphane direct from absolute alcohol or cleared in xylene and mounted in balsam.

Amoebae undergoing nuclear division have considerable depth at all of the mitotic stages. When they are placed on a slide they tend to become flattened if the surrounding water is partially removed. Further flattening is obtained by dropping the fixative directly on the animal from a pipette. This flattening facilitates the process of staining and destaining, as well as later study. Since, however, the possibility exists that the cytoplasmic movements involved in the flattening process might cause distortion of the spindles, few slides were made by this method. The majority of the animals studied were either dropped directly into the fixative, or were flooded with fixative before a detectable change in shape occurred.

Development and Behavior of the Spindle

Mid-prophase

Spindle fibers first make their appearance during mid-prophase. At this time the nuclear membrane is intact, and the nucleus appears as a flattened sphere, measuring 22×19 microns. The chromosomes, which are numerous and small, have become loosely grouped on a plate measuring 18×14 microns in polar-view. The chromosomal fibers are fairly distinct, lying approximately at right angles to the chromosome plate, and measuring four microns in length.

Late-prophase

At a slightly later stage of prophase, first described for the giant amoeba by Kudo (1947), and similar to a stage described and pictured by Liesche (1938) for

Amoeba proteus, the chromosomes are well oriented on a plate measuring 13 × 11 microns, and the nuclear membrane is sharply contracted until it is in contact with the ends of the chromosomal fibers. The fibers are four to five microns long, and are definitely, although not greatly arched. The entire nucleus in edge-view appears as a slightly rounded rectangle. Kudo (1947) in his description of this stage states that "the spindle fibers undergo further development and extend to the nuclear membrane." This is not in agreement with the observations of the writer. The fibers do not appear to increase appreciably in length, but rather the nuclear membrane contracts until it is in contact with the fibers.

Metaphase

The metaphase figure presents much the same picture as the late prophase stage described above, but the nuclear membrane has now disappeared, and the chromosome plate is more compact, measuring 11 × 9 microns. The arching, or curvature, and length of the chromosomal fibers show no appreciable change.

Anaphase-telophase

In an unflattened amoeba containing many nuclei, the anaphase or telophase figures are found distributed throughout the animal. They are found at all focal levels with their long axes vertical, horizontal and tilted at various angles. There are significantly more side-views than polar-views, as might be expected, since the depth of the animal is less than its other dimensions, but in other respects the distribution of figures is a random one. In some regions few figures are seen, while in others many may be found in close proximity. In an amoeba 60 microns thick, and containing many food vacuoles, some anaphase or telophase figures will inevitably be partly obscured, and perhaps a few entirely hidden. The spindles of strictly polar-views were not possible to analyze, and the same was true of some part-polar-views and obscured side-views (Table I).

Many different degrees of daughter chromosome separation were studied, involving more than forty amoebae. Distances between daughter groups varied from less than one micron to sixty-five microns. The appearance of the figure changes considerably as the groups of daughter chromosomes move progressively farther apart. Early stages continue to show the arching of chromosomal fibers observed at late prophase and metaphase, but this curvature is gradually lost. The chromosomes of each daughter group become increasingly closely aggregated on the plates. As the plates decrease in diameter the chromosomal fibers become more closely packed and straight. The outside chromosomal fibers at late anaphase are inclined at a sixty degree angle to the plate. They do not change materially in length but remain four to five microns long throughout division.

Early anaphases show the interzonal fibers as straight lines from plate to plate. Later, however, when the chromosome plates have moved farther apart, there is a pronounced barreling of these fibers—the diameter of the interzonal spindle being considerably greater at its center than its diameter close to either plate.

The appearance of granules along the ends of the chromosomal fibers has been taken to be the beginning of telophase since it is along the region of these granules that the daughter nuclear membrane forms (Short, 1946). If this is used as a criterion for the beginning of telophase, then there is an overlapping of anaphase and telophase stages with respect to the length of interzonal fibers. Granules at the

ends of chromosomal fibers and even developing nuclear membranes have been observed when the groups of daughter chromosomes were as little as 20 microns apart, while more often groups much farther apart show no evidence of granules or membrane. When the new membrane has completely formed, both chromosomal and interzonal fibers disappear.

The fibers of the interzonal spindles, which are barreled, at mid-anaphase lie approximately parallel to one another. This barreling of the interzonal spindle clearly indicates that its elongation is meeting with resistance. Occasionally figures are seen in which daughter groups of chromosomes have encountered obstacles to their movement. In one such instance two anaphase figures, elongating in the same plane, met end to end. The two sets of chromosomal fibers which collided were crumpled, and interzonal fibers of both figures were buckled. Similar effects were observed in cases in which one or both groups of chromosomal fibers encountered a food vacuole or the pellicle. It seems evident that the pushing forces of elongation continue even when further separation of groups of daughter chromosomes is prevented.

When interzonal spindles reach a length of 18 to 20 microns, barreling of the fibers disappears. The fibers now appear relaxed. Accompanying this apparent relaxation is a pronounced and consistent right-hand twisting of the interzonal spindle. In no amoeba studied at or beyond this stage, were the interzonal spindles either straight or twisted to the left (Table I). A very few spindles showed the

TABLE I

Frequency of interzonal spindle twisting in two amoebae

Distance between plates—microns	Number of figures analyzed			Number twisted to right	Number twisted to left
	No. 1	No. 2	Total		
1–14	0	0	0	0	0
15–17	1	1	2	2	0
18–22	6	47	53	53	0
23–27	16	19	35	35	0
28–32	13	2	15	15	0
33–37	8	1	9	9	0
38–42	6	1	7	7	0
43–47	1	0	1	1	0
Analyzed	51	71	122	122	0
Undetermined*	55	37	92		
Total	106	108	214		

* Polar, semi-polar, and 27 side-views partially obscured.

right-hand twist when only 15 microns long. These were in animals in which most spindles were considerably longer. Elongation of the spindle continues and the twisting persists. The longest interzonal spindle which could be analyzed measured 45 microns, and showed a distinct right-hand twist. Many spindles of greater length were observed, the longest measuring 65 microns. These invariably had a rope-like appearance, and individual fibers could not be distinguished.

Several amoebae which showed twisted spindles did not show enough figures for statistical study. Two amoebae were selected which contained a large number of figures which could be accurately analyzed. Data obtained from these two animals are presented in Table I. The two animals contained a total of 122 spindles which it was possible to anaylze. These varied in length from 15 to 45 microns. All of these spindles were twisted to the right.

Discussion

Kudo (1947) discusses the reports of Short (1945, 1946), Dawson, Kessler, and Silverstein (1935), Hinchey (1937), Liesche (1938) and Nozawa (1939) of twisting of interzonal spindle fibers at late anaphase in various amoebae. He offers the simple explanation that "the inter-plate fibers become twisted because of the violent boiling movement of the protoplasm. . . ." Unfortunately he seems to have missed the point stressed by Short that "the fact that the spindles consistently twist in one direction indicates that this twisting which accompanies spindle elongation is not a chance phenomenon brought about by cytoplasmic movement or other external factors operating on the spindle." The same reasoning that Kudo uses in criticizing a statement of Hinchey would seem to apply in this case. In commenting on Hinchey's statement that "protoplasmic streaming next moves the plates apart," he makes the well taken point that "if this is the case, the streaming must necessarily be localized and the direction of streaming must be opposite for each of the hundreds of dividing nuclei, which cannot be verified." It seems equally unreasonable to suppose that "boiling" protoplasm could produce a twisting of spindles constant in direction.

Dawson, Kessler and Silverstein (1935) report spindle twisting in *Amoeba dubia* at mid-telophase. They state that "almost invariably a twisting of the spindle occurs at this stage." These workers unfortunately do not comment on the direction of twisting, but it is interesting to note that their illustration entitled "Typical twisting of spindle cylinder" shows a right-hand twist.

There are a number of striking similarities between the appearance of the spindle at various stages of division in the giant amoeba, and those described for the uninucleate *Amoeba fluvialis* by Dobell (1914). The chromosomal fibers of both animals are short, become more closely packed as division progresses, and do not change appreciably in length throughout division. Interzonal fibers of both animals are straight at early anaphase, barrel out as anaphase progresses and appear to relax at late anaphase or early telophase. It is noteworthy in this connection that while Dobell merely states that the spindles at this stage are "often somewhat twisted," his figure illustrating the stage likewise shows a distinct right-hand twist.

Hinchey (1937), in describing anaphase movement in the giant amoeba, states that "the chromatin then separates into two plates which move along the spindle fibers toward the poles until the plates are three times their diameter apart." It seems difficult to explain how the chromosome plates can "move along the spindle fibers." The writer is in agreement with the following statement of Dobell (1914) in reference to *Amoeba fluvialis*. "It seems certain that the daughter groups of chromosomes are separated from one another by the growth of the spindle fibers lying between them—not by the chromosomes moving along the

spindle fibers; for the cap-like ends of the spindle remain of the same size during the whole process, whilst the fibers between the chromosome groups become longer and longer." Such a pushing apart of the groups of daughter chromosomes by the elongation of the interzonal fibers seems the only logical conclusion to be drawn from the barreling of these fibers at mid-anaphase and their buckling when obstacles to elongation are encountered.

When the extreme elongation of the interzonal fibers is considered it seems improbable that they arise from a stretching of the chromosome pellicle or sheath, as has been suggested by various workers on other material (Schrader, 1944). The evidence here seems to be all in support of a "pushing" hypothesis. It is suggested that the development of a definite fiber between centromeres, or the poles, might bring about the separation of the metaphase chromatids and that its continued elongation might account for the further separation on the part of the daughter chromosomes.

The constancy of the direction of the twist of the interzonal spindle must be determined by the molecular structure of the fibers. The molecular organization of these fibers may well be in the form of a coil, which when uncoiled would permit a twist in only one direction, accompanied by relaxation. The hypothesis of a molecular spiral has been proposed by Darlington (1935) to explain twisting of paired chromosomes about each other as they elongate at meiotic prophase. If lateral attractions between interzonal fibers exist, then the principles involved in Darlington's hypothesis for chromosomes would apply to the twisting of the spindles.

The work of Dawson, Dobell and other investigators mentioned above suggests that twisting of the interzonal spindle might be found to be a consistent phenomenon in species other than the giant amoeba. Amoebae are considered to be especially favorable material for a study of this phenomenon. Spindles of many organisms, being confined by small cell limits, may never reach a length that allows twisting to become apparent. A greater range of organisms, in which a considerable elongation of the spindle is possible, should be investigated for evidence of the twisting here described.

Summary

The chromosomal fibers of the giant amoeba appear during prophase, approximately at right angles to a loosely organized chromosome plate. At late prophase they are more closely grouped and slightly arched. This arching is observed during metaphase and early anaphase, the fibers becoming straight at mid-anaphase. They do not change materially in length at any stage of nuclear division. Chromosomal fibers become increasingly closely packed as the diameter of the chromosome plates decreases.

Interzonal spindles are straight at early anaphase, barrel out at mid-anaphase, and appear relaxed at late anaphase and telophase. They reach a length of 65 microns, but spindles more than 45 microns long appear rope-like and individual fibers cannot be distinguished. When groups of daughter chromosomes with their chromosomal fibers encounter an obstacle to their movement, chromosomal fibers are crumpled, and interzonal fibers buckle. This behavior, and the barreling of mid-anaphase spindles, must be interpreted as evidence that anaphase separa-

tion in this organism consists of a pushing apart of the groups of daughter chromosomes by the elongation of the interzonal spindle.

Interzonal spindles 18 or more microns long are consistently twisted to the right. Uncoiling of a spiral of constant direction within the interzonal fiber, accompanied by a lateral attraction between fibers, is offered as a possible explanation of the constancy of the direction of the twist.

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CALCIFICATION IN MARINE MOLLUSCS *

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Introduction

The correlation between mantle activity and the production of shells in molluscs has been of interest for a considerable time. Studies by Rawitz (1888, 1890) have shown in considerable detail the histological structure of the mantle tissues of a number of species. Biederman (1914) and Schmidt (1923) described the morphological aspects of shell formation with particular reference to the pattern and arrangement of the various components. The reports of Moynier de Villepoix (1892), Brooks (1905), Hass (1935), Bourne (1943) and others too numerous to mention have also added considerably to our understanding of the structure, composition, crystalline pattern and in some degree to the detailed mechanism involved in the calcification of the shell.

Despite a voluminous literature (see Hass, 1935) which has appeared in connection with calcification in molluscs, several phases of this problem still await further study and clarification. By means of methods to be described in the following paragraphs we have examined in detail a number of aspects of shell formation and in so doing have utilized several different methods of attack upon the problem.

Material and Methods

The species of molluscs used in this investigation consisted of *Atrina rigida* (*Pinna*), *Pedalion alatum*, *Codokia orbicularis*, *Pinctata radiata*, and *Venus mercenaria*. For studies in a closed system the young spat of *Pedalion* proved to be most successful. For rapid regeneration studies in which a glass cover slip was placed in the mantle cavity (Brooks, 1905) *Pinna* proved to be efficacious. *Codokia* and *Venus* were better suited for histological and histochemical studies than other forms examined due to the relative lack of pigment in the mantle tissues.

Standard techniques and procedures were used for histological studies. Mucin was localized by Hoyer's thionin method, phosphatase, pre-formed phosphate and reticulum according to the methods of Gomori (1937, 1941), and calcium according to the method of Feigl (1943). The artificial sea water media used in our experiments was made up according to the formula of McClendon (Rogers, 1927).

In this study we were concerned with various factors relating to calcification. It was therefore necessary to choose a number of different test animals in order that different aspects of the problem could be investigated. For certain phases of the study, histological and histochemical observations were made, for others a change in the environment of the animal was initiated, in a third set of observa-

* This work was supported in part by a grant-in-aid contributed by Dr. Harry Seldin.

tions calcification was induced on a glass cover slip (Brooks, 1905), while still another set of observations were obtained by means of time-lapse photography.[1]

In order to stimulate growth of the shells in several species, a portion of the shell was removed. By means of this procedure, we were able to obtain rapid regeneration and observe a number of details related to shell deposition in a relatively short time.

Observations

For an understanding of the processes which will be described later it seems appropriate at this juncture to indicate briefly the salient morphological features of the mantle of the mollusc. Essentially, the mantle consists proximally of a thin sheet of tissue which is applied to the inner surface of the shell, while distally the mantle breaks up into a number of tentacle-like folds usually three in number. During the process of shell formation these folds undulate back and forth in the region of the free margin of the shell. The greater part of the mantle is covered by epithelium which varies in character from low cuboidal to the high columnar form. Mucous glands are very numerous, widely distributed and contain granules which give a positive test for calcium phosphate.

In some respects the formation of the shell is reminiscent of the early histogenesis of membrane bone. There are two distinct processes which occur: (1) the elaboration of a fibrous organic membrane, and (2) the concentration and deposition of mineral salts.

The elaboration of the organic matrix is brought about by the activity of a layer of cuboidal cells occupying the outer surface of the middle fold of the mantle (Figs. 1 and 3). The secretion elaborated by these cells is known as conchin or periostrachum. This substance, first deposited as a thin sheet, comes to be arranged in prisms and striae in later development (Fig. 6) and according to our own and other observations (Hass, 1935) is a protein and contains one or more reducing sugars. When first elaborated it takes a positive reticulum stain, later it undergoes certain modifications in staining reactions; the prismatic portion is acidophilic, the inner portion exhibits basophilic properties.

The various steps in the process of calcification of the shell were studied advantageously by means of the "cover-glass" technique. As shown in Figures 7 and 8, the first observable indication of mineralization consists of a deposit of numerous minute granules which appear to be embedded in the surface of the conchin layer. According to our observations these granules consist of calcium phosphate, and preliminary tests[2] reveal the presence of both inorganic and organic forms.

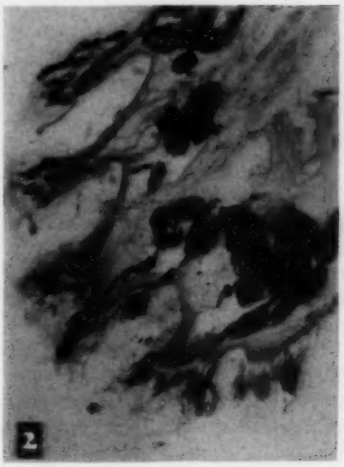
If one recalls that the mucous tissue is extremely abundant and widely distributed in the mantle tissues and that the mucous is secreted at the surfaces of the epithelial cells (Fig. 2), it becomes readily understandable that when the tentacles of the mantle undulate back and forth on the newly formed conchin layer, the granules will naturally be deposited on its surface and adhere thereto.

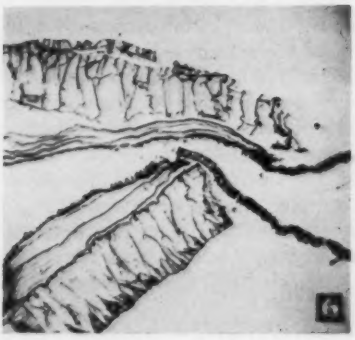
Once the granules are deposited in the conchin layer, they begin to undergo a typical crystal growth such as is shown in Figures 9 and 10. Continued growth

[1] Courtesy Mr. Edward Baylor, Princeton University.

[2] Tests made by Miss Lowell Lowell.

PLATE I





eventually results in an arrangement whereby the crystals (in *Pinna*) come to be enclosed in a thin layer of organic matrix and assume a polyhedral shape (Fig. 11). These crystals are composed of calcite (Biederman, 1914; Hass, 1935).

One interesting cytochemical feature which was observed in this connection was the identification of a strip of epithelium covering the surface of the inner middle fold which contains heavy concentrations of the enzyme alkaline phosphatase (Fig. 5).

Effect of Modifying Sea Water

Although lack of time did not permit a detailed study of the effect on calcification due to changes in the constituents of sea water, it was nevertheless possible to record a few pertinent observations in regard to this matter.

Using McClendon's formula for artificial sea water, young specimens of normal Pedalion were placed in this media; it was observed that they were alive and in good condition after a period of several days. Following this a number of specimens which had the free edge of the shell cut back as far as the mantle tissues were placed in this same media. Figure 12 is a photograph showing normal shell growth (regeneration) in a typical specimen.

Having shown that apparently normal calcification takes place under the conditions of the experiment, calcium was withdrawn in varying amounts from the artificial sea water and the resultant effects were observed in several specimens. The results of the experiments in which calcium was partially removed from sea water are shown in Figures 14, 15. Figure 12 shows a normal regenerating shell grown in sea water with the usual amount of calcium present. Figure 14 shows a specimen in which 50 per cent of the calcium was withdrawn, while Figure 15 illustrates a specimen grown in sea water containing only one-eighth of the normal calcium content. This latter specimen is completely devoid of calcium in the newly formed shell. Interestingly enough, however, the organic matrix was deposited in an apparently normal manner.

A few experiments were also made in which the magnesium content of the sea water was modified. Reference to Figure 13 shows that a similar condition may obtain when this mineral is withdrawn.

Discussion

In our studies concerning the calcification of the shell in several marine molluscs we have reviewed the essential histological structure of the mantle tissues and in so doing find that we are in all important respects in agreement with the reports of

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Figure 1. Longitudinal section of mantle of *Codokia*, hematoxylin and eosin showing formation of conchin.

Figure 2. Section of mantle of *Codokia* stained with thionin to show secretion of mucous.

Figure 3. Section of mantle of *Venus*, hematoxylin and eosin. General topography of folds and elaboration of conchin.

Figure 4. Mantle of *Venus* to show distribution of mucous. (Thionin)

Figure 5. Mantle of *Venus* showing distribution of alkaline phosphatase.

Figure 6. Decalcified section of shell of *Pedalion* showing arrangement of organic part of shell. Strand of tissue on right is regenerating tissue.

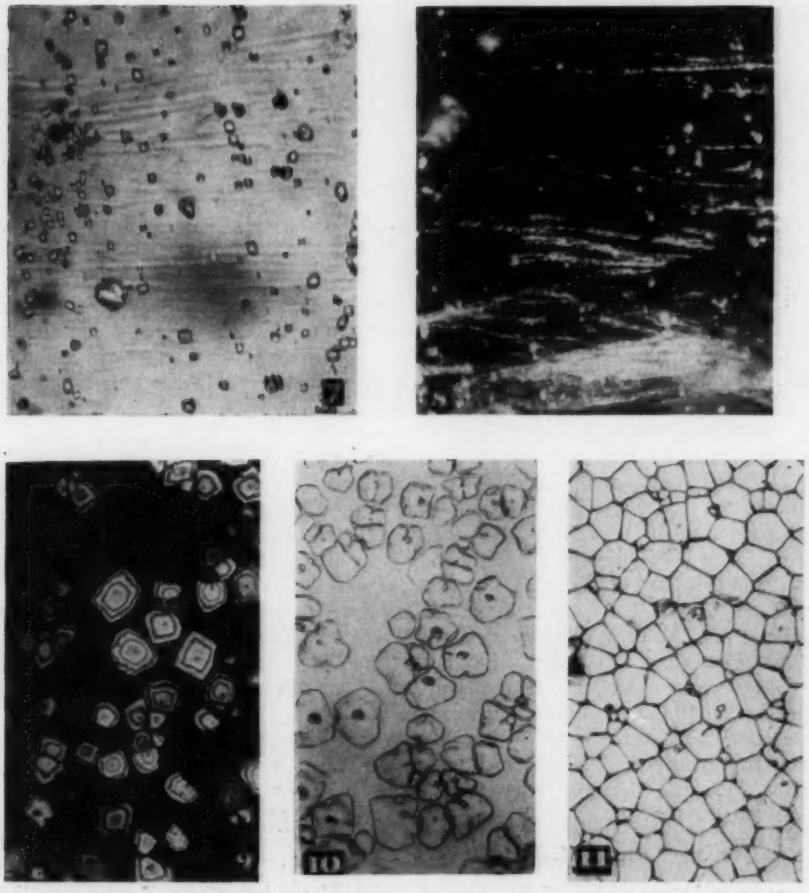


PLATE II

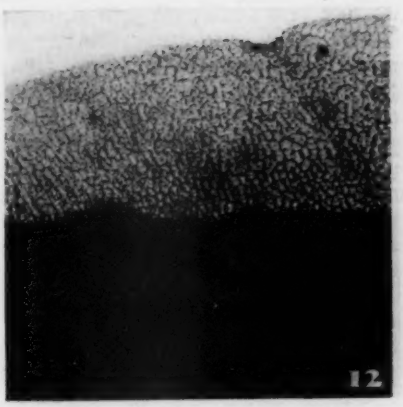
FIGURE 7. First formed elements of shell of *Pinna* deposited upon a cover slip. Note granules and fibrous matrix. Photo by transmitted light.

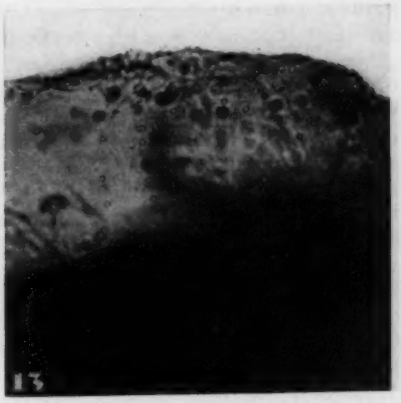
FIGURE 8. Similar preparation photographed by means of oblique illumination.

FIGURE 9. Crystal growth on cover slip preparation (*Pinna*). Photographed through crossed Nicols prisms.

FIGURE 10. Similar preparation photographed by means of transmitted light.

FIGURE 11. Mature shell (*Pinna*) surface view.





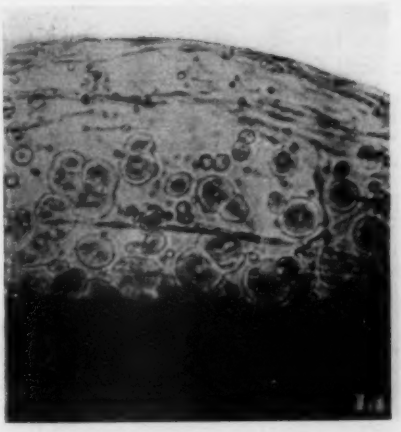


PLATE III

FIGURE 12. Normal regeneration of shell of young *Pedalion* in artificial sea-water.
FIGURE 13. Regeneration of *Pedalion* shell in which Mg was reduced.
FIGURE 14. Regenerating shell in 50 per cent normal Ca content.
FIGURE 15. Regenerating shell in one-eighth normal Ca content.

the earlier investigations (Rawitz, 1888, 1890). The observations of Biederman (1914), Schmidt (1923) and others in reference to the morphological aspects of shell formation have also been confirmed in part by our own observations.

In regard to the more minute details which occur during the process of calcification, Biederman (1914) refers to several pertinent observations and reports that while mantle tissue contains calcium phosphate in abundance, the calcified shell is made up of calcium carbonate and is completely lacking in phosphate. A similar statement appears in Plate (1922). Biederman further poses the interesting question concerning the mechanism of the transfer of phosphate to carbonate. Upon this point Moynier de Villepoix (1892) made some conjectures which seem to us at this time to have been extremely discerning: he states that it might be permissible to suppose that after the deposition of the granules in the conchin, that they might serve as the center of attraction of the elements and that the crystal growth of the calcium carbonate be determined in some manner thereby.

In regard to the presence of phosphatase on the surface epithelium of the mantle tissue, this condition was reported by Bourne (1943). Although this author's description is in many respects in agreement with our own observations, we are not certain that his photograph in fact indicates the presence of phosphatase, for our observations reveal that phosphatase is present on the surface of the cells which come in contact with the matrix and the granules, and not deeply embedded in the cytoplasm as indicated by Bourne.

In spite of the fact that many details have been omitted in this description of the process of calcification in the mollusc shell, it seems nevertheless feasible to suggest, if only in outline form, the essential steps which are involved:

An organic matrix is elaborated by epithelial cells located on the surface of the middle mantle fold. This substance gives a positive test for protein and reducing sugars. It also undergoes certain changes in arrangement and tinctorial properties as it matures.

The first observable anlage of the mineral part of the shell arises in the mucous glands, it is composed of calcium phosphate and comes to be deposited upon the matrix (conchin) due to the surface contact of the folds which pass over this tissue when the shell is being formed. The final calcified product is composed of calcium carbonate (calcite).

How this calcium phosphate is converted to the final calcified product, calcium carbonate, can be only conjectured at this time.[3] It does not seem likely that the conversion is a direct reaction between calcium phosphate and the carbonate of sea water for this process would require exceedingly high concentrations of carbonate.

Greenwald (1938) has emphasized the role played by other ions, particularly organic ones, in increasing the solubility of calcium phosphate. Such ions presumably act by binding calcium in some manner, possibly in an un-ionized moiety of the molecule. It is therefore proposed that the calcium phosphate may be dissolved by the participation of some organic ion. Phosphatase may aid this process by transferring phosphate to some substrate, thus removing the phosphate ions which otherwise would tend to slow the formation of the calcium complex. In a

[3] Freeman and Wilber (1948) have demonstrated the presence of carbonic anhydrase in mantle tissue and body fluids of some pelecypods and gastropods. However, these authors carefully point out that while it may have importance in shell formation in some species, negligible activity in others indicates that shell may be deposited in its absence.

further phase of this process, calcium ions may be made available by the alteration of the organic ion possibly through an oxidation-reduction reaction. If high local concentrations of calcium ions were thus made available, the carbonate of the medium would slowly precipitate calcium carbonate resulting in the crystalline architecture we have noted in our investigations.

Summary

1. Various aspects of the process of calcification have been studied in several marine molluscs.

2. Calcification of the shell is brought about by the formation of an organic matrix upon which minute granules of calcium phosphate are deposited.

3. In the presence of, and in contact with the mantle epithelium, crystal growth occurs in sea water.

4. The enzyme alkaline phosphatase appears to be concerned with calcification in these forms.

5. Modification of certain constituents of sea water results in the production of a shell partially or completely lacking in mineral content.

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REGENERATION IN PEROPHORA VIRIDIS[1]

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The potency of the Ascidians to regenerate and their susceptibility to environmental agents have long been known. Driesch (1902) found that small isolated portions of Clavellina stolon could form complete individuals. Huxley (1921) studied dedifferentiation in Perophora and later (1926) dedifferentiation in Clavellina. Berrill and Cohen (1936) studied the regeneration of isolated pieces of Clavellina stolon with respect to the polarity of the parent zooid.

Perophora, a colonial Ascidian, because of its relative structural simplicity and the uniformity of its growth pattern, seemed to offer excellent material for regeneration experiments, especially with respect to the polarity relationships of isolated portions to the colony as a whole.

The experiments to be described were carried out with the following objectives: (a) To investigate the regeneration of isolated stolons; (b) to study the regeneration of stolon tips; (c) to test the regeneration of stolonic material when expressed from the tunic; (d) to determine the effect of ligatures on isolated stolons; (e) to study the regeneration of stolon-zooid systems.

The author wishes to acknowledge the helpful guidance of Dr. Lester G. Barth during the course of this investigation.

Material and Methods

The experiments were performed chiefly upon freshly collected Perophora colonies. Satisfactory stock material was also cultured by suspending "grape-like" clusters of colony by string in a tank of running sea water. In three or four days new stolon outgrowths, which could be handled conveniently, appeared. The experimental animals were kept in separate syracuse dishes which were immersed in a large glass aquarium through which filtered sea water constantly flowed.

A Perophora colony consists of a system of zooids appearing at fairly regular intervals from a branched stolon (Fig. 1, A). The zooids show a size seriation, becoming progressively smaller toward the free end of the colony. This seriation is due to the mode of origin of the zooid, for as the stolon grows in length buds appear near the free end and develop into mature zooids as the colony grows. Thus, the size seriation is an index of the age of the zooids, the largest zooids always being the oldest. The free growing end of the stolon in the experiments to be described will be called the distal end, as opposed to the attached end which is called proximal.

As described by Deviney (1934), the stolon of Perophora is cylindrical, its

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wall being composed essentially of three layers. It consists of an outer tunic with scattered cells plus intercellular substance, a middle epithelio-ectodermal layer which is quite thin, and an inner discontinuous thin layer of mesenchyme. The mesenchymal layer lines the stolonic cavity through which blood flows; this cavity is divided vertically by a single-layered septum. The septum disappears near the distal end of the stolon, allowing continuity of blood flow between the right and left halves of the cavity.

The operations and observations were made under a binocular microscope. All cuts were made by means of a pair of iridectomy scissors. Photographs were taken with an Argus camera, attached to a compound microscope and drawings were made from these photographs.

Results and Discussion

A. Regeneration of isolated stolons

In these experiments the portion of stolon lying between two zooids was excised by two cuts and observed for regeneration (Fig. 1, B). The excised stolons varied in length from 2.5–6.0 millimeters. Either distal or proximal ends of the excised stolon were stained with nile blue sulphate suspended in agar, for purposes of orientation. The results are listed in Table I.

Table I

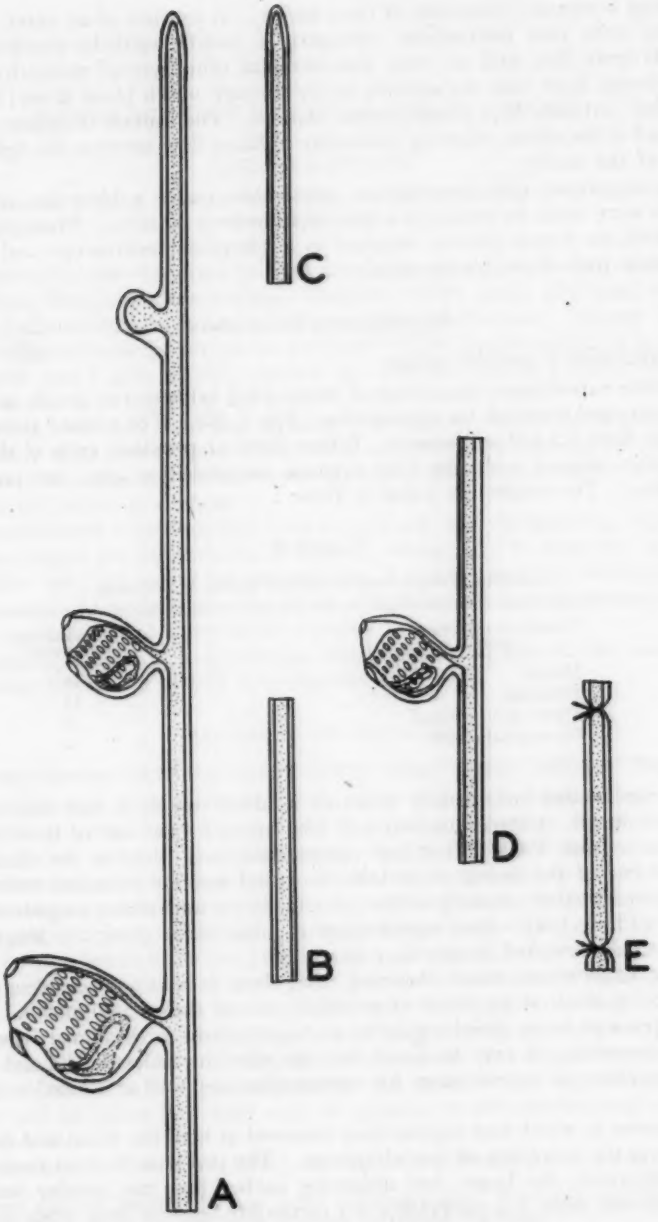
Location of regenerants in excised stolons of Perphora

Location of regenerated zooid	No. of specimens regenerated
Distal	18
Proximal	11
Distal and proximal	9
On original stolon	4

The regenerated buds usually appeared in about two days, and reached an approximate length of three hundred and fifty micra by the end of three days. It can be seen from Table I that bud regeneration may occur at the distal or the proximal end of the stolon, or at both the distal and the proximal ends. These buds appeared either precisely at the cut end, or on new stolon outgrowths at the cut end (Pl. I, 1–4). Bud regeneration at some point along the length of the original stolon occurred in only four cases.

These experiments show, therefore, that there is a definite tendency for regeneration to occur at the distal or proximal ends of the excised stolon. Further, there appears to be no definite polarity as regards distal or proximal regeneration. In this connection, it may be noted that the nile blue sulphate did not have an inhibitory effect on regeneration, for regeneration occurred at stained ends of the stolons.

The cases in which bud regeneration occurred at both the distal and the proximal ends of the stolon are of special interest. The two buds in most cases showed a size difference, the larger bud appearing earlier than the smaller bud. This may mean that there is a competition for metabolite between buds when more than


C
D
B
E
A

one is involved in regeneration. The smaller bud is less successful in this competition and develops more slowly. In one case, the smaller bud was eventually resorbed at the expense of the larger bud. That a competition between two regenerating regions exists is further evidenced by the fact that when bud formation occurred at only one end, there was frequently a preliminary stolon growth at the other end of the excised stolon. This stolon growth was eventually resorbed and there was a general drawing of the original stolon materials towards the developing bud (Pl. I, 2, 4).

B. Regeneration of stolon tips

The portions of stolon used in this experiment consisted of distal pieces. The stolon was isolated by a cut made just distal to the first visible bud (Fig. 1, C). Of eleven such stolon pieces, varying in length from 1.7 to 4.8 millimeters, all formed buds in one to two days. These buds appeared in approximately the same position on the stolon as do new buds in normal colony formation (Pl. I, 5). No buds were observed at the proximal end of the isolated stolon, though in five cases small stolon outgrowths did appear. In two of these five cases the proximal growth was later resorbed at the expense of distal growth.

There is thus a difference in the mode of regeneration of isolated stolon tips as compared with that of stolon pieces isolated from between two zooids. In the former, the growing distal tip is probably more active in development at the time of the cut; after the cut is made, this lead is maintained. In the latter case, both ends are cut and their development may proceed on a more nearly equal basis as regards competition.

C. Regeneration of stolonic material expressed from the tunic

Wilson (1923) attempted to test the regenerative power of mesenchyme cells which he pressed out of the stolon. He obtained clumps of cells which stuck together, but which died within a day. Since, as shown by Deviney (1934), in the budding of *Perophora viridis,* the unspecialized cells of the septum and of the blood stream are the formative elements, it seemed likely that with more favorable conditions the stolonic material might be kept alive, and its ability to regenerate tested.

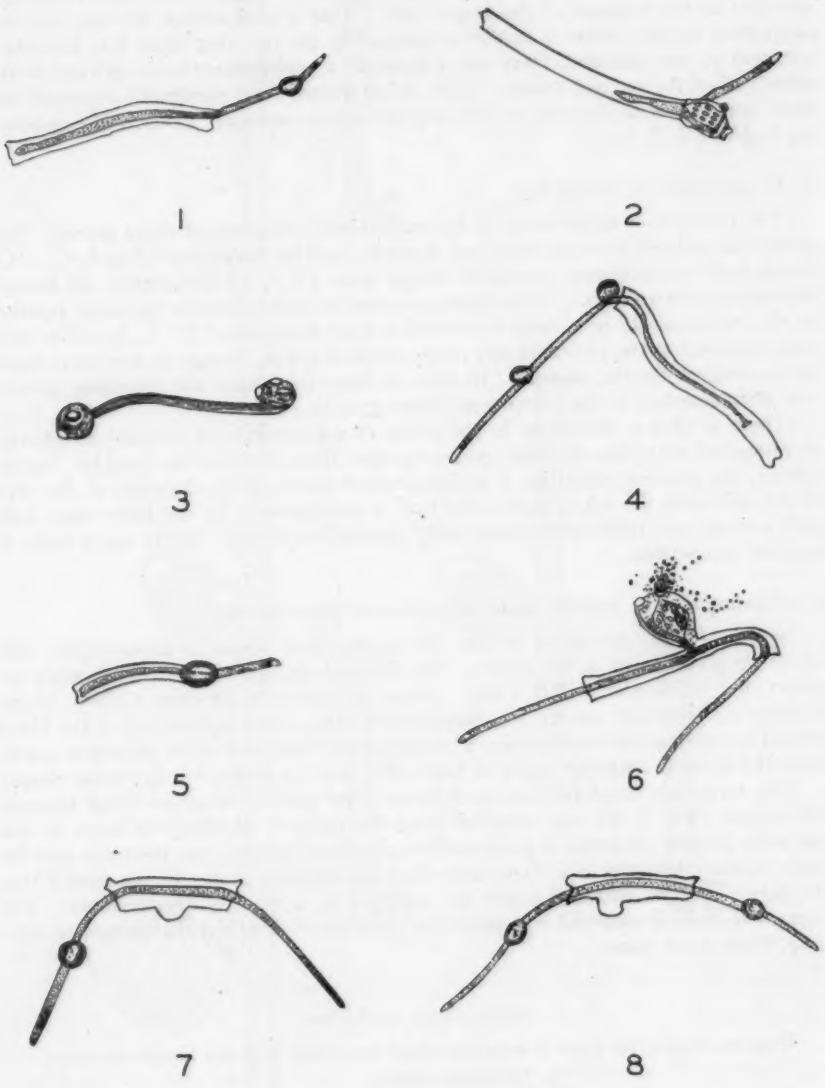
The technique employed was as follows: The portion of stolon lying between two zooids (Fig. 1, B) was removed from the colony. Holding the tunic at one end with jeweler's forceps, a glass needle was passed gently over the tunic and the inner stolonic material was expressed from the opposite end. It was found that the inner stolonic material could be removed as a unit in this manner. The expressed stolonic material was placed in syracuse dishes and maintained in running, filtered sea water.

EXPLANATION OF FIGURE

Diagram to show the types of isolations which were made from the Perophora colony.

A. Perophora colony.
B. Stolon isolation.
C. Stolon tip isolation.
D. Stolon-zooid system.
E. Stolon ligatured at both ends.

PLATE I



Of a total of nineteen such cases, nine showed stolon growth and bud formation after three days, while ten showed stolon growth but no bud formation. A new tunic was observed around the stolonic materials at this time. The point of origin of new buds showed marked variation.

Since the expressed material contained the unspecialized cells of the septum and of the blood stream, it is not surprising that under favorable environmental conditions the material could survive and form buds. Wilson (1923) assumed that the material expressed from the tunic consisted of mesenchyme cells. However, Deviney (1934) has shown, as cited earlier in this paper, that there is a thin epithelio-ectodermal layer surrounding the stolonic cavity. It is possible that cells of the epithelio-ectodermal layer were included with the expressed material in these experiments, so that it may not be concluded that a particular cell type was solely responsible for bud formation. However, it is suggested that, by this method, particular cell types may be isolated, and their ability to regenerate studied.

D. Ligature of isolated stolons

The thread ligature as a means of isolation was first employed by Morgan (1902), and later by Peebles (1931) and Barth (1938 a), on Tubularia. In Tubularia the perisarc remains intact after the ligature, but the coenosarc is severed, thus isolating the stem on each side of the ligature. The thread ligature acts in a similar way in Perophora. The tougher tunic is not cut, but the inner stolonic tube is effectually severed by the ligature.

Twenty-five stolon pieces, each of which was isolated from between two zooids, were ligatured at each end by means of fine cotton threads (Fig. 1, E). Twenty-two specimens consisting of similarly isolated stolon pieces, but without any ligatures, were used as controls. The results of the experiment are summarized in Table II. The ligature hindered stolon growth and bud formation, while typical stolon growth and bud formation occurred in the controls. It may be that in the one instance that regeneration occurred in the ligatured stolon, it was because the ligature was so tight that it cut through the tunic.

Barth (1938 b) has shown that the availability of oxygen is an important factor in controlling the rate of regeneration. It is suggested that in Perophora

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EXPLANATION OF PLATE I

FIGURES 1–8 (×12)

1–4. Types of regenerants obtained after isolation of stolons removed from between two zooids. 1. Bud formation on a stolon outgrowth from the distal end of the stolon, four days after isolation. 2. Zooid formation at the distal end of a stolon, six days after isolation. 3. Bud formation at both ends of a stolon, after five days. 4. Stolon growth and bud formation at the proximal end of an isolated stolon, after five days.

5. Bud formation on an isolated stolon tip, after two days.

6–8. Types of growth and regeneration obtained after isolation of stolon-zooid systems. 6. Growth of the zooid and stolon growth at both ends of the stolon, after 6 days. 7. Resorption of the zooid, stolon growth at both ends of the stolon, and bud formation on the proximal stolonic outgrowth, after 6 days. 8. Resorption of the zooid, stolon growth at both ends of the stolon, and bud formation on both stolonic outgrowths, after 6 days.

TABLE II

The effect of thread ligature on stolon and bud regeneration

	Time in days	No. of specimens	Bud regeneration with stolon growth	No bud regeneration	
				Stolon growth	No stolon growth
Ligatured	5	25	1	3	21
Control	5	22	11	4	7

there is a lowering of the availability of oxygen at the cut ends brought about by the thread ligatures, thus inhibiting regeneration.

E. Regeneration of stolon-zooid systems

By isolating a normal zooid along with a short length of stolon from the Perophora colony, Huxley (1921; 1934) demonstrated a differential susceptibility of the stolon-zooid system. When the stolon-zooid system is starved under normal conditions, the stolon is resorbed by the zooid. When the stolon-zooid system is placed in dilute toxic solutions, the zooid is resorbed by the stolon. This may be interpreted as meaning that under normal circumstances there is a competition between zooid and stolon, the zooid being dominant because of a higher metabolic rate. Under moderately unfavorable conditions, the metabolically more active zooid is more severely effected, and the stolon assumes the dominant role. Thus, there is a delicate equilibrium between zooid and stolon. That region which is more active grows at the expense of the other region.

Since running sea water seems to offer the most favorable environment for survival of Perophora material, it was thought advisable to isolate stolon-zooid systems (Fig. 1, D) and place them in running filtered sea water. Presumably, under these conditions, food is available; and it was felt that further information might be obtained on the competitive relations of stolon to zooid. Each specimen was kept in a separate syracuse dish and immersed in the container through which the sea water flowed.

For the thirty-four stolon-zooid systems isolated, the results fell into three classes:

(a) The original zooid was maintained, and actually increased in size in many cases. In addition, there was stolon growth at either or both cut ends, and buds formed on these outgrowths. This occurred in thirteen cases (Pl. I, 6).

(b) The original zooid was resorbed, there was stolon growth at either or both ends, and bud formation occurred on these outgrowths. There were twenty such cases (Pl. I, 7, 8).

(c) The zooid grew at the expense of the stolon, the latter being resorbed. This occurred in only one of the thirty-four cases.

From these experiments it would appear that under the most favorable conditions, i.e. running sea water plus healthy stolon and zooid, the entire stolon-zooid system may grow. The stolon grows and may form buds, and the original zooid may maintain itself and even increase in volume. Evidence of feeding in healthy

zooids under these environmental conditions has been noted (Pl. I, 6). Rather than assume a competition between stolon and zooid, from the experiment as run in this fashion, it would seem more plausible to assume that a competition occurs between three growing regions, namely the zooid and the two cut ends of the stolon. Under the most favorable conditions, when all three regions are developing equally rapidly, all continue to grow. When one gets ahead in development, it may develop at the expense of one or both of the others.

That there is this competition is demonstrated in the cases where stolon growth occurred at only one end, for in eight of these cases, it first occurred at both ends, but was later followed by stolon resorption at one end, and further stolon growth at the other end. Of the three growth zones in the isolated stolon-zooid system, the zooid appears to be most sensitive, having been resorbed in twenty of the thirty-four cases.

This explanation may be considered, then, as an extension of Huxley's ideas on equilibrium and competition, rather than as a negation of his fundamental assumptions.

General Discussion

The results of these experiments are interesting in view of those obtained with Tubularia. Both Tubularia (Goldin and Barth, 1941) and Perophora have relatively unspecialized cells which have the ability, under the proper environmental conditions, to reconstitute the whole individual. In the normal colony formation of Perophora, buds do not normally form on the stolon lying between two zooids. In Tubularia, similarly, a hydranth generally does not form between the distal hydranth and the proximal end of the stem. A cut in either Perophora or Tubularia provides an environmental stimulus which initiates the process of regeneration at the point of the injury. Barth (1938 b) has suggested "that the stimulus for regeneration is the exposure of tissue to high oxygen." Goldin (1942 a; 1942 b) has shown that both oxygen and hydrogen ion concentration influence the rate of regeneration of Tubularia. Increased oxygen stimulates regeneration, while increased hydrogen ion concentration has an inhibitory effect on regeneration. That in Perophora, too, similar mechanisms are involved, is supported by the following evidences: In the isolation of stolons excised from between two zooids, and of stolon-zooid systems, regeneration occurs at the cut ends or on stolonic outgrowths at the cut ends. In Perophora regeneration is hindered when both ends of the stolon are ligatured, this inhibition probably being correlated with the lowered availability of oxygen and increased concentration of acid metabolites brought about by the thread ligature. Finally, when stolonic material is expressed from the tunic, providing ready diffusion of oxygen and metabolites, regeneration may occur at any point along the axis of the expressed material.

It has been shown in Tubularia that there is a definite polarity present in the stem. After isolation, distal levels exercise dominance over more proximal levels and the distal end regenerates more rapidly. In Perophora, stolons isolated from between two zooids show no evidence of original polarity relationships in regeneration. Regeneration may occur at either end or both ends, with equal frequency. In Perophora, proximal regeneration unaccompanied by distal regeneration represents a reversal of polarity, and is in marked contrast to the situation in Tubularia.

Despite this failure to maintain the original distal-proximal polarity, however, once regeneration has begun, a new competitive physiological axis is set up without any relation to the original distal-proximal orientation. This competition has been manifested in the following way: In isolated stolons regenerating new stolonic material at both ends, one end may often cause resorption of the stolonic growth at the other end. A similar situation may occur when buds form at both ends of the isolated stolon, the larger bud eventually causing resorption of the smaller bud. In isolated stolon-zooid systems there exist three active regions, namely, two cut ends, and a zooid. More rapid growth at any one of these may progress at the expense of other regions. It is interesting to note that under optimum conditions, competition is eliminated and growth occurs at all of these regions.

That factors other than oxygen availability and diffusion of metabolites are involved in the regeneration of Perophora stolons is demonstrated by experiments in which distal stolonic tips are isolated. In these stolon tips there is probably greater oxygen availability and diffusion at the cut proximal end. Despite this fact, regeneration and growth occurs at the distal end.

It is suggested that, as with Tubularia, Perophora offers a good biological system for studying, further, problems of regeneration, polarity, and differentiation.

Summary

1. Stolons of Perophora, isolated from between two zooids, regenerate at the cut ends but do not necessarily maintain their original distal-proximal polarity relationships.

2. Stolon tips, after isolation, maintain their distal-proximal polarity, the distal end displaying dominance.

3. The inner stolonic material, when expressed from the tunic, may grow and regenerate buds at any point along its axis.

4. Regeneration is hindered at ligatured ends of stolons.

5. When stolon-zooid systems are isolated and maintained under optimum conditions, the entire stolon-zooid system may grow.

6. The theoretical implications of the results of these experiments are discussed.

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HISTOLOGY OF THE CORPORA ALLATA OF MELANOPLUS DIFFERENTIALIS (ORTHOPTERA: SALTATORIA)

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The corpora allata of insects are known to secrete a hormone called "juvenile" or "inhibitory" hormone which is responsible for maintaining the insect tissues in a juvenile condition and therefore retards metamorphosis (Wigglesworth, 1934, 1936, 1940; reviews by Hanström, 1939; Scharrer, 1941, 1948; Bodenstein, 1942; Piepho, 1943; Joly, 1945a; Mendes, 1947). They also play a role in egg production and in the development or function of male and female accessory glands (Wigglesworth, 1936; see reviews). Whether the different physiological effects of the secretion of the corpora allata are brought about by one or more than one hormone is still a controversial matter. The grasshopper *Melanoplus differentialis* has been the subject of experimental study by Pfeiffer (1939, 1945, 1945a) who has shown that hormone from the corpora allata retards metamorphosis and in adult females controls certain metabolic phenomena, the production of oviducal secretion and the deposition of yolk. It was therefore chosen for study of the histology of the corpora allata at different periods of the life history in an attempt to correlate the histological aspects with the experimental data.

Material and Methods

Melanoplus differentialis passes through six nymphal stages before metamorphosis. Histological preparations were made of the corpora allata of male and female nymphs which were killed at daily intervals during the fifth and sixth stages to compare the changes in these glands during a stage that precedes the production of nymphal characters at molting with those occurring prior to metamorphosis, and to determine whether there is any sexual dimorphism in the nymphal glands. Corpora allata from adult males and females killed at significant ages were prepared for comparison of these glands in the two sexes, for comparison with nymphal glands and for study of the histological changes in adult female corpora allata in relation to egg production.

The grasshoppers used in this study were reared as described by Pfeiffer (1945a). The corpora allata were dissected from freshly cut heads submerged in insect saline. For control purposes some of the corpora allata were dissected from heads not immersed in saline; they showed no difference from those dissected when submerged. The ages of the dissected grasshoppers were recorded in terms of the number of days that had elapsed since the preceding molt. The approximate length of the intermolt periods was learned from control nymphs of the same

[1] The author wishes to express her sincere thanks to the staff of Osborn Zoological Laboratory, Yale University, for the kind hospitality she has received while doing the present research. To Dr. I. W. Pfeiffer she expresses her gratitude for having introduced her to the field of insect endocrinology.

molting dates. When the corpora allata were dissected from adult females, the presence or absence of oviducal secretion and yolk was noted, the oocytes nearest the oviducts were measured, and the blood color was recorded. Several fixatives were tried. Of these, Susa's technique gave the best results and therefore was used almost exclusively. The glands were imbedded in paraffin. The sections, seven to eight micra in thickness, were stained with hematoxylin and eosin, toluidin blue and erythrosin, methylene blue and erythrosin, Masson's trichrome stain (hemalum, erythrosin and safranin) and Foot's modification of Masson's stain (Weigert's hematoxylin, Ponceau de xylidine, acid fuchsin and light green).

I owe to Dr. I. W. Pfeiffer the privilege of studying iron hematoxylin slides which were useful for the counting of chromosomes.

Topography and Morphology

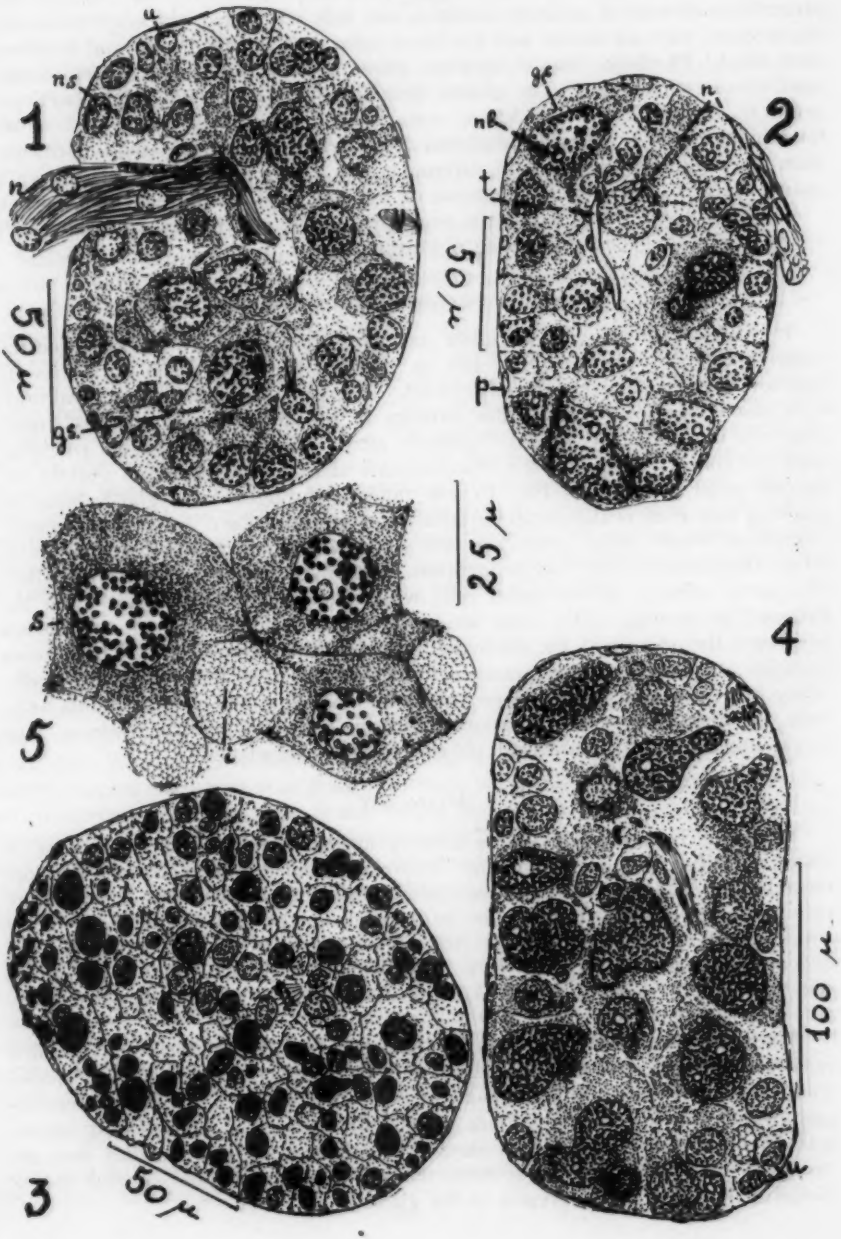
In *Melanoplus* the corpora allata are paired subspherical structures located ventro-laterally to the pharynx, just in front of the crop. The aorta has its anterior opening at about the same level. Therefore, the glands are suspended in a blood lacuna into which the contents of the dorsal vessel are discharged. They are innervated by the nervi corporis allati. There are no nerves emerging from the corpora allata. The nervus corporis allati enters through a slightly depressed point (the hilum, Fig. 1) and ramifies throughout the gland, branches reaching almost all of the glandular cells. Covering the gland is a delicate connective tissue sheath which penetrates the glandular tissue at the hilum and is in direct continuation with the perineurium that covers the nervus corporis allati. The nerve contains a few small cells with spherical nuclei different from the flattened perineurium cells; both kinds are found inside the gland. A trachea penetrates the organ with the nerve, and tracheal cells enter with it. This trachea branches abundantly, so that numerous tracheoles are to be found among the cells. Many fine tracheae may also be seen along the surface of the gland at points away from the hilum. They vary in number with the age of the animal, tracheae being more numerous on the surfaces of old or hypertrophied glands.

Histology

General.—The structure of the corpora allata is fundamentally the same in males, females, nymphs and adults. However, sexual differences and developmental stage and age variations are sufficiently well defined that sex and approximate age of the animal can be estimated from the histological aspect of the corpora allata. Four types of cells may be recognized in the corpora allata: (1) connective tissue cells (of the outer sheath and the perineurium) and tracheal cells; (2) undifferentiated cells; (3) normal secretory cells; and (4) giant secretory or polyploid cells.

The undifferentiated cells (Figs. 1, 4 and 8) are small spherical or polyhedric cells, about 10 to 14 micra in diameter, with relatively large spherical nuclei. These cells have only a small amount of cytoplasm and do not contain secretory granules or vacuoles. They divide mitotically, exhibiting the greatest mitotic activity at the beginning of each developmental stage. However, they may occasionally be found in mitosis at other times during the nymphal or adult stages. Undifferentiated cells are present in the gland at all times.

PLATE I



The normal secretory cells (Fig. 1) may be distinguished from the undifferentiated cells only during active secretory stages of the gland. They vary in volume in accordance with the phase of the cycle of activity of the individual cells. They may become strongly polymorphic, at which time the nucleus is large, usually more or less lobulated, with abundant deeply staining chromatin; one nucleolus is always present. Under the conditions of fixation employed, the cytoplasm of the normal secretory cells is dense and acidophil. During active phases it has a granular appearance due to the presence of strongly acidophil granules. The granules appear first around the nucleus and gradually increase in quantity. When they have become numerous, intracellular vacuoles appear (Fig. 9). These vacuoles vary in size. They may become very large, occupying nearly the entire space at one side of the nucleus. In cells located near the periphery of the gland, the vacuoles are frequently situated on the side of the nucleus toward the center of the gland, under which conditions the cytoplasm in the outer portions of the cell may contain closely crowded acidophil granules. In some cases the content of the vacuoles appears as evenly dispersed granules. In others, particularly the larger ones, it has coagulated into a network. The staining reaction of the vacuolar contents is faintly acidophilic.

When intracellular vacuoles are numerous, vacuoles that are similar in appearance may also be seen between the cells (Figs. 5 and 8), suggesting that secretory material has passed out of the cells into intercellular spaces. At this time, also, particularly when the appearance of the gland suggests the most intense secretory activity, numerous fine lines which suggest "lines of flow" may be seen following somewhat radial courses from central regions of the gland to the periphery. They are especially noticeable in the outer portions of the gland (Fig. 9). When viewed with changing focus, they frequently appear to be between the cells since they are seen most distinctly before the nuclei and cytoplasm come into clear view. At the level of the nucleus the cytoplasm has the usual aspect of evenly

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EXPLANATION OF FIGURES

1. Corpus allatum of fifth instar female nymph killed within 24 hours after molting. The four types of cells are recognizable. Acidophil granules are present in the secretory cells. No intra- or intercellular vacuoles have yet been formed. Mitoses are present. *gs*, giant secretory cell; *n*, nerve entering at the hilum; *ns*, normal secretory cell; *u*, undifferentiated cell.

2. Corpus allatum of fifth instar male nymph killed 8 days after molting. The secretory cells are beginning to decrease in size. Acidophil granules are scarce. A few intercellular vacuoles are still present. The giant secretory cells have polymorphic nuclei. They are more numerous in proportion to the number of normal secretory cells than in female corpora allata (compare with Fig. 1; see also Fig. 4). *gs*, giant secretory cell; *n*, nerve; *nl*, nucleolus; *p*, peripheral connective tissue cell; *t*, trachea.

3. Corpus allatum of sixth instar male nymph killed 2 days after molting. The different types of cells are hardly distinguishable. The cell membranes are distinct. Mitoses are numerous. No secretory material is present.

4. Corpus allatum of sixth instar male nymph killed 4 days after molting. Giant secretory cells with polymorphic nuclei are numerous. Acidophil granules are present near the nuclei. No intercellular vacuoles have as yet been formed. Occasional mitoses are still present. *u*, undifferentiated cells.

5. Three normal secretory cells from a corpus allatum of a sixth instar female nymph killed five days after molting. Intercellular vacuoles are present. Cell and nuclear membranes are indistinct in places. *i*, intercellular vacuole; s, acidophil granules.

dispersed granules and intracellular vacuoles. Sometimes, however, the acidophil substance in the outer regions of the peripheral cells may itself be disposed in lines perpendicular to the surface of the gland, thus contributing to the general aspect of outwardly radiating lines.

While the secretory granules are appearing, the nuclei of the normal secretory cells often become swollen and vacuolated, and the nuclear membranes become more delicate, until in some places no boundary between nucleus and cytoplasm can be distinguished (Figs. 5 and 8). However, acidophil granules were never found in the nucleus. When the nucleus acquires the swollen aspect, chromatin-like granules are usually found in the cytoplasm near it (Fig. 5). Mitoses were never found in secretory cells that had enlarged sufficiently to be distinguished from undifferentiated cells.

The giant secretory cells (Figs. 1, 2 and 8) are polyploid cells which arise from nuclear mitoses not followed by cytoplasmic division. The nucleus is two to four times larger than the nuclei of normal secretory cells. It is polymorphic, never spherical; its chromatin stains deeply, and it usually contains two to four nucleoli. When the nucleus is undergoing polyploid mitosis, the quantity of cytoplasm is about the same as that of normal diploid cells at rest, but thereafter it expands until the cytoplasmic volume is proportional to the chromatin content. As the cytoplasm becomes more abundant, the cell bodies tend to acquire an increasingly irregular shape. However, in every respect except size the giant secretory cells are similar to the normal ones. During the secretory phase acidophil granules appear in the same concentration as in normal secretory cells. Intracellular vacuoles develop. The nuclear membrane becomes gradually less distinct, and chromatin-like granules are frequently found in the cytoplasm. Most of the giant cells are located in the central portion of the gland, and during stages of intense secretory activity the gland, in sections through its center, may tend to have a stratified appearance (Fig. 8) with a central region containing giant cells and areas of dense cytoplasm, a more peripherally located region rich in intra- and intercellular vacuoles and an outer region in which the nuclei of secretory cells and undifferentiated cells are often arranged side by side to form a border. Due to cellular enlargement and the formation and growth of vacuoles, the gland expands during secretory activity.

Polyploid cells were found in which the chromosome number, counted in the equatorial plate, was between 90 and 100. The cells resulting from such mitoses must be octoploid since the normal diploid number for *Melanoplus differentialis* is 24 (King and Slifer, 1934). This large number of chromosomes was not very often encountered, but tetraploid cells are common in the corpora allata. In a few instances the number of polyploid mitoses in the corpora allata of recently molted nymphs was counted and compared with the number of giant cells in glands of the same nymphal stage which had passed the period of strong mitotic activity. This counting was most conveniently done on sixth instar corpora allata since here it is easy to know when the peaks of cell division and secretory activity are reached. A total of 5 polyploid mitoses, one of them octoploid, was counted in a series of 5 corpora allata from females examined 0 to 4 days after molting; therefore one in each gland. After the fourth day no more polyploid mitoses were found. From 3 to 8, or an average of 3.6 giant cells per gland, were found in females that had spent 5 to 7 days in the sixth stage.

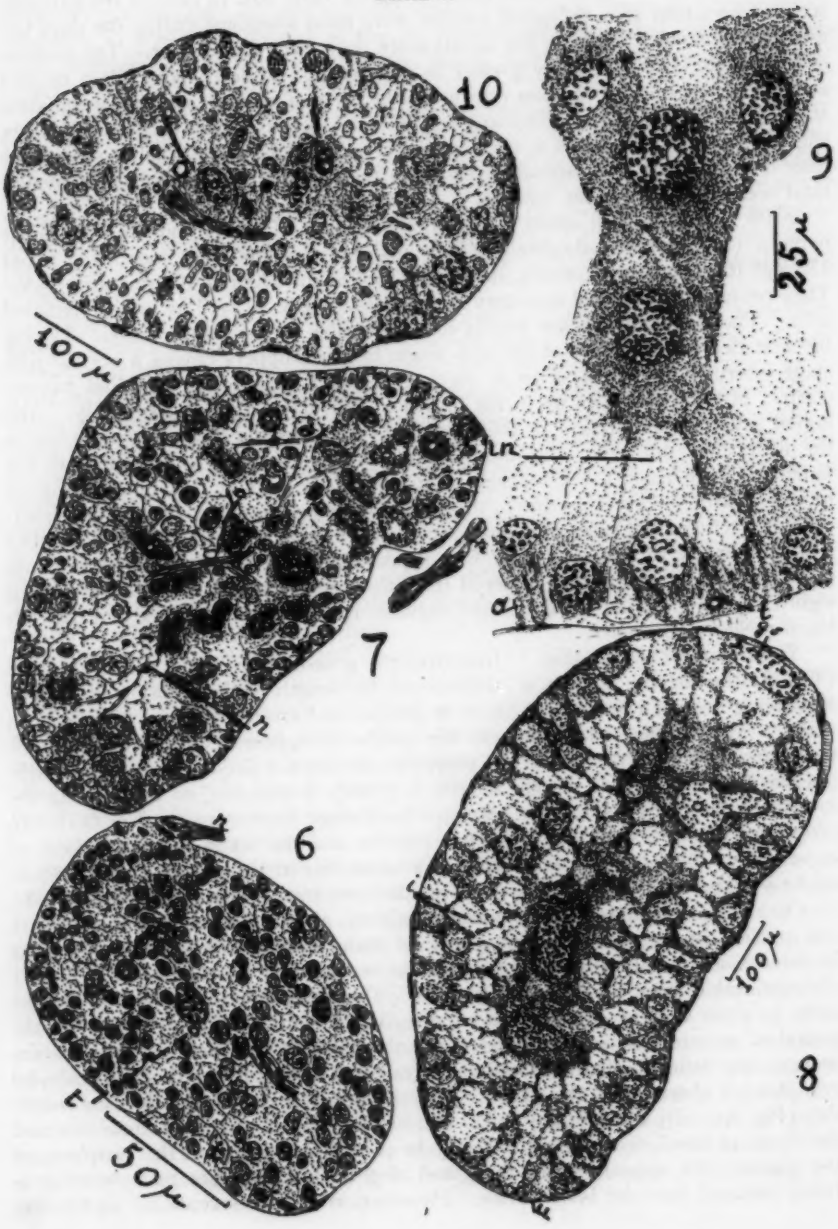
Polyploid mitoses were found only during the early part of each of the nymphal stages. In adults also, polyploid mitoses were most abundant during the days immediately following molting, but occasionally they were found later. The number of giant cells is progressively greater in each stage. Male corpora allata contain about the same number of giant cells as do the corpora allata of females of the same stage, but they are more prominent in the males because male corpora allata are slightly smaller and contain fewer normal secretory cells. The smaller size of the male corpora allata is presumably related to the fact that the males have a smaller total body size than do the females.

Fifth instar corpora allata. The corpora allata of newly molted fifth instar nymphs (Fig. 1) are subspherical, their major and minor axes measuring about 150 and 100 micra, respectively, in females, and about 120 and 100 micra in males. They contain both normal secretory and polyploid cells, as well as undifferentiated cells. The polyploid cells are readily distinguishable by their large nuclei. During the first two or three days of the fifth stage, in grasshoppers having a normal fifth stage intermolt period of seven to eight days, both diploid and polyploid mitoses occur. Mitosis continues up to the fourth to fifth days but less frequently. Acidophil granules are present in the secretory cells from the day the nymph enters the fifth stage until the next molt occurs. They are most abundant from the fourth to sixth days, the cytoplasm of the secretory cells at this time having an intensely acidophil reaction due to the closely crowded granules. Intra- and intercellular vacuoles are also abundant during the fourth to sixth days. The latter tend to increase in size after their first appearance, and the gland shows evidence of active release of secretory material from the fourth day on. By the seventh or eighth day (Fig. 2) both granules and vacuoles become less numerous, and the cells begin to decrease in size.

Sixth instar corpora allata. Just after the grasshoppers enter the sixth stage (Fig. 3) the corpora allata are subspherical, the length of their major and minor axes being around 180 and 130 micra in females and around 150 and 120 micra in males. At this stage all of the cells are similar in appearance, resembling undifferentiated cells. Both normal and polyploid mitoses are present, and immediately after molting the rate of mitotic activity is greatly accelerated, so that during the first three days, in grasshoppers having a sixth stage intermolt period of twelve to fifteen days, one to four and even five mitoses may be found in each section of eight micra. Mitoses are somewhat more numerous in females than in males, as might be expected since the male corpora allata are smaller and contain fewer cells.

On the second and third days after molting, although numerous mitoses are still present in the gland, the cytoplasm of many of the cells begins to increase in volume, and on the third and fourth days normal and giant cells are definitely distinguishable from undifferentiated cells. The cytoplasmic phenomena are the same in sixth stage corpora allata as described for fifth instar nymphs, but the period of secretory activity is briefer and the steps of the secretory process are more clearly defined. During the first two or three days of the sixth stage acidophil granules are absent from the gland. They begin to appear on the third or fourth day (Fig. 4). By the fifth day intra- and intercellular vacuoles are numerous and the "lines of flow" described earlier may be seen directed toward the periphery of the gland. The appearance of the gland at this time suggests that secretion is being released into the body cavity. However, from the seventh or eighth day

PLATE II



until the end of the sixth stage the gland shows no signs of active production of secretion, and mitoses are infrequent. During this stage the giant cells shrink in both nuclear and cytoplasmic volume until it becomes impossible to tell them from normal diploid cells (Fig. 6).

Adult female corpora allata. In newly molted adult females the corpora allata are somewhat flattened bodies, usually roughly circular in outline. Their major and minor axes measure about 400 and 200 micra, respectively. At this time all of the cells are small and similar in appearance, as they were at the close of the sixth stage. However, immediately after molting, in grasshoppers developing at a rate such that oviducal secretion can be expected to appear on the seventh or eighth day of the adult stage, the corpora allata begin to enlarge rapidly, due to cell proliferation, to cytoplasmic expansion and to increase in nuclear volume (Fig. 7). During the first four or five days of the adult stage both normal and polyploid mitoses are present. They are found frequently until the first group of oocytes reach 0.6 mm. in length, but decrease in number during the period when the oocytes grow from 0.6 to 0.75 mm. By the time the production of oviducal secretion and yolk begins mitoses are rare, although they may occasionally be seen throughout the remainder of the adult stage, presumably to replace worn out cells.

Acidophil granules are lacking at the time the female enters the adult stage, but they may be present in some of the secretory cells by the second day after molting if development is rapid. Intra- and intercellular vacuoles may be present by the third or fourth day, with the gland having the appearance that release of secretion to the body cavity has begun by the fourth day. By the fifth or sixth day the gland may have the appearance of intense secretory activity, with the nuclei and cytoplasm greatly expanded, intra- and intercellular vacuoles large and the "lines of flow" strongly evident. The adult corpora allata usually show considerably greater expansion during active phases than is usual for the nymphal corpora allata.

After the secretory phase of the adult corpora allata has begun, it continues at a high level (Fig. 9) throughout the sexually active life of the female. The cytological and histological aspects remain the same and show no variation with blood color changes or with the egg development cycles. When the adult female

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EXPLANATION OF FIGURES

6. Corpus allatum of sixth instar female nymph killed 8 days after molting. Almost all of the cells look alike. Acidophil granules and vacuoles are absent. *t*, trachea.

7. Corpus allatum of adult female killed 2 days after molting. The four types of cells are readily recognizable. Acidophil granules are beginning to appear. *n*, nerve.

8. Corpus allatum of adult male killed 15 days after molting. The gland is at the height of secretory activity. Acidophil granules and vacuoles are present. The gland has a stratified appearance due to the distribution of the granule-filled cytoplasm and the vacuoles. *gs*, giant secretory cell with vacuolated nucleus, nuclear membrane indistinct in places; *i*, intercellular vacuoles; *u*, undifferentiated cells.

9. Portion of the outer region of a corpus allatum of an adult female killed 26 days after molting. The flow of secretory material toward the outside is shown at the periphery of the gland. *in*, intracellular vacuole; *o*, "lines of flow"; *t*, trachea.

10. Corpus allatum of an adult female killed 60 days after the last molt. The first signs of senescence are present. Acidophil granules are becoming scarce. Connective tissue is becoming more prominent both in the outer membrane and within the gland.

becomes old the amount of secretion in the corpora allata gradually diminishes, the outer membrane increases in thickness and the cytoplasm is stained with more difficulty (Fig. 10). Mitoses are absent and the gland decreases somewhat in size.

Adult male corpora allata. Except for their smaller size, the fewer normal secretory cells and the prominence of the giant cells, the corpora allata of adult males (Fig. 8) are histologically very similar to those of adult females developing at the same rate. The corpora allata of newly molted males are usually roughly circular in outline and somewhat flattened. The cells are small, resemble one another, and are devoid of acidophil granules. During the early part of the adult stage there is a period of cell proliferation and nuclear and cytoplasmic expansion. Both normal and polyploid mitoses occur frequently during the first three or four days after molting, then gradually decrease in number. As the cells expand, acidophil granules and then intra- and intercellular vacuoles appear. The corpora allata attain full secretory activity at about the same time in males as in females, and they show the same great expansion of the nuclei and cytoplasm during the active phase.

Discussion

Evidence from experimental studies supports the concept that in hemimetabolous insects (*Rhodnius*, Wigglesworth, 1934, 1936, 1940; *Dixippus*, Pflugfelder, 1937, 1937a, 1939; *Leucophaea*, Scharrer, 1946), as well as in holometabolous insects (*Bombyx*, Bounhiol, 1939), juvenile hormone is released by the corpora allata during the post embryonic stages which precede molts that are accompanied by the development of juvenile (nymphal or larval) characters but is absent or ineffective during the stage which precedes the production of adult characters and metamorphosis. The tissues are evidently stimulated to develop juvenile characters during a "critical period" in the nymphal (or larval) stage when juvenile hormone attains effective concentrations in the blood. That development of the grasshopper *Melanoplus differentialis* conforms to this principle has been suggested by the results of experimental studies (Pfeiffer, 1945) and is now further confirmed by the histological findings of the present investigation. It was seen in this regard that secretory material, presumably juvenile hormone, is elaborated in the corpora allata throughout the greater part of the fifth (next to last) nymphal stage and is most actively released during a period that begins around the middle of the stage and continues until shortly before molting. The "critical period" during which the insect is caused to develop the nymphal characters that appear at the end of the fifth stage may, therefore, be supposed to occur during the latter half of this stage.

On the other hand, the corpora allata of *Melanoplus* also show evidence of secretory activity in the sixth stage, a fact which suggests that juvenile hormone is again being produced. If this is true, it must be concluded that the hormone released in this stage is ineffective in inducing the development of nymphal characters, since metamorphosis occurs at the end of the stage. A reason for such ineffectiveness is suggested by the fact that the entire period of elaboration and release of secretory material in the sixth stage is brief; it does not begin until the second or third day after molting and is completed around the middle of the stage. It would therefore seem probable that the amount of hormone produced is inadequate, or its release may not take place at the proper time to prevent meta-

morphosis. There is also the possibility, suggested by evidence from other insects (*Drosophila,* Bodenstein, 1943; Vogt, 1943; *Leucophaea,* Scharrer, 1946), that the tissues may be less responsive to juvenile hormone in the sixth than in earlier stages, a condition which would further decrease the effectiveness of such amounts of hormone as are produced. In regard to these possibilities, Pfeiffer (1945) has shown that metamorphosis can be delayed in *Melanoplus,* and nymphal characters caused to appear at the end of the sixth stage, by supplying additional amounts of corpus allatum hormone.

Wigglesworth (1934) has also described histological evidence of secretory activity in the corpus allatum of *Rhodnius* during the last (fifth) nymphal stage but was unable by experimental means to detect the presence of juvenile hormone in the blood. On the basis of recent experiments (1947) he has postulated that the corpora allata of old fifth stage nymphs or young adults of *Rhodnius* cause removal of juvenile hormone from the blood. No evidence of a similar function has been observed in the histology of the corpora allata of *Melanoplus.* However, that hormone from the corpora allata may fall to low concentrations in the blood or disappear at the end of the last nymphal stage in this insect, and that it does not regain high levels until about the time the influence of the corpora allata on adult functions (Pfeiffer, 1939, 1945a) is manifest, is suggested by the histological evidence. The corpora allata, it was seen, show no evidence of secretory activity during the last six or seven days of the sixth stage, or on the day of entering the adult stage, but achieve the histological appearance of strong secretory activity shortly before the production of oviducal secretion and yolk begins. Pfeiffer (1945a) has shown in this connection that metabolic conditions in females of *Melanoplus* during the early part of the adult stage are like those continuously in effect when the corpora allata have been removed, but change when there is sufficient hormone in the blood to induce the production of oviducal secretion and yolk.

It is of interest that after the corpora allata have attained high levels of secretory activity in adult females, they continue functioning at such levels, showing no histological evidence of cyclic changes related to the egg production cycles. Joly (1945) likewise found no histological evidence of intermittent activity of the corpora allata of *Dytiscus* but reasoned from experimental evidence that the corpora allata function cyclically in adults of this insect. Also of interest is the fact that the corpora allata of adult males of *Melanoplus* show evidence of as intense secretory activity per unit of glandular tissue as do those of adult females, suggesting that the concentration of hormone in the blood may be maintained at similar levels in both sexes. The smaller size of the corpora allata in the males seems to be related to the smaller total body size of the males rather than to a functional sexual dimorphism. Considering that the corpora allata have a number of functions related to reproduction in adult females of *Melanoplus* (Pfeiffer, 1939, 1945a), but have so far not been found to have comparable importance in males, it could have been expected that they would show a lower level of activity in the males. The histological evidence would therefore seem to support the idea, which has been gaining increasing favor (Pflugfelder, 1938a; Day, 1943; Pfeiffer, 1945a), that the various functions of the corpora allata (see reviews by Hanström, 1939; Scharrer, 1941, 1948; Bodenstein, 1942; Joly, 1945a; Mendes, 1947) are accomplished, in part at least, through effects on metabolic processes rather than through specific actions on the different tissues involved.

In regard to whether the corpora allata secrete different hormones to perform different functions, it can only be said from the present observations that no evidence could be detected histologically that more than one hormone is produced either simultaneously or in the different stages. On the other hand, the fact that the corpora allata of adult grasshoppers may assume an appearance of more intense secretory activity than is seen in nymphs presents the possibility that the concentration of hormone from the corpora allata may reach higher levels in the blood of the adults and that this may have some significance in connection with the different functions of the corpora allata in nymphs and adults. It has been suggested (Bodenstein, 1943; Scharrer, 1946) that differences in hormone concentration and responsiveness of the tissues may play an important part in this connection.

The corpora allata have been described, both topographically and histologically for a large number of insects, the most comprehensive study in this regard having been made by Nabert (1913). References to the many recent descriptions may be found in papers by Hanström (1942); Poulson (1945) and Mendes (1947). The histological characteristics of the corpora allata of *Melanoplus* agree in a number of respects with those described for other insects. Among these may be mentioned: the acidophil reaction of the secretory granules; irregular shape of the cells and nuclei under certain conditions; abundance of chromatin; and the presence of large nucleoli. The occurrence of fine fibrils or striations which may constitute the same phenomenon as that described as "lines of flow" in the present study has been reported to occur in the corpora allata of termites (Pflugfelder, 1938) and in *Gryllotalpa* (De Lerma, 1932). Vacuoles are usually mentioned as a characteristic of active corpora allata. However, Wigglesworth (1934) did not find vacuoles in the corpus allatum of *Rhodnius* when the gland was active, but instead saw them between the cells when the latter had shrunk during a period of inactivity. In *Melanoplus*, it was seen, intercellular vacuoles were present at the height of secretory activity.

It is a general characteristic of the corpora allata that they expand during secretory activity and shrink during inactivity. The expansion may be due simply to enlargement of the cells or, as has been seen in *Melanoplus*, to both cellular expansion and the presence of large vacuoles. There may also be differences in the fundamental size of the gland which are related to the body size of the insect and are based on differences in the number of cells in the gland. In *Melanoplus*, it was seen that the corpora allata are larger and contain more cells in the females than in the males, which in all stages are, on the average, smaller individuals than are the females. The corpora allata also becomes progressively larger in each developmental stage. In agreement with other hemimetabolous insects (*Rhodnius*, Wigglesworth, 1934; *Dixippus*, Pflugfelder, 1937), the latter growth takes place during a period of cell proliferation which occurs at the beginning of the developmental stages. *Rhodnius* differs from *Melanoplus* and *Dixippus*, however, in that there is no proliferative phase at the beginning of the adult stage (Wigglesworth, 1934).

The cells of the corpora allata have generally been described as approximately alike, the principal variations being those shown by all of the cells of the gland in relation to activity and inactivity. However, in the corpora allata of fourth instar nymphs of *Rhodnius*, Wigglesworth (1934) distinguishes between centrally located cells which become swollen and acidophil during the critical period, and periph-

erally located, dividing and growing cells which do not become acidophil. These differences suggest a distinction between undifferentiated cells and secretory cells similar to that seen in *Melanoplus*. De Lerma (1932) found giant cells in *Gryllotalpa* which may be comparable to those observed in *Melanoplus* and which he describes as arising from the fusion of several cells. In *Melanoplus*, on the other hand, it was seen that the giant cells are the result of polyploid mitoses. Since these mitoses occur during the early part of each developmental stage, when mitotic activity is most intense and when the gland is entering an active secretory phase, it seems unlikely that they represent a degenerative condition. Instead, the fact that they have a large volume of cytoplasm in which is elaborated secretory material that cannot be distinguished histologically from that produced by the normal secretory cells suggests that they may be a means of increasing the secretory effectiveness of the gland. It is of interest that the giant cells make up a greater proportion of the gland in males then in females.

It seems probable that the polyploid chromosome content of the giant cells is the result of two or more mitoses of the nuclei of undifferentiated cells taking place in rapid succession. This hypothesis is supported by the fact that the volume of cytoplasm in these cells remains small until division of the nucleus is completed, suggesting that the nuclear divisions are not separated by periods of cytoplasmic expansion. The normal secretory cells, on the other hand, evidently arise from normal diploid division of the undifferentiated cells. This process occurs principally during the proliferative period in each developmental stage, but may occur at other times. Since undifferentiated cells are present in the gland at all times they constitute a reserve from which additional cells may be produced during the time the gland is in the secretory phase. They are also carried over from one stage to the next and are probably, in part at least, the cells in which the new wave of mitotic activity originates. It is possible, however, that the normal secretory cells may also undergo mitosis when they are reduced in size during inactive periods.

Summary

1. The corpora allata of the grasshopper *Melanoplus differentialis* were studied histologically to determine whether these glands show changes related to their functions in the control of nymphal development and the production of ripe eggs and oviducal secretion.

2. Four classes of cells were recognized in the corpora allata of this insect: a, connective tissue and tracheal cells; b, undifferentiated cells; c, normal secretory cells; and d, giant secretory or polyploid cells.

3. The undifferentiated cells are small cells which divide mitotically and do not contain secretory granules or vacuoles. They show the greatest mitotic activity at the beginning of each developmental stage but occasionally divide at other times.

4. The glandular cells, both normal and polyploid, show characteristic changes related to secretory activity and inactivity which are fundamentally the same in nymphs and adults and in both sexes. During inactive phases they are reduced in size and resemble undifferentiated cells. During active phases they increase in cytoplasmic and nuclear volume, the cytoplasm becomes filled with strongly

acidophil granules, then intracellular and finally intercellular vacuoles appear. At the height of secretory activity the appearance of the gland suggests that materials are flowing from central regions of the gland to the periphery.

5. In the fifth nymphal stage, at the end of which nymphal characters are produced, secretory material is elaborated throughout the intermolt period; active release of secretion occurs during the latter half of the stage. In the sixth stage, which terminates with the production of adult characters and metamorphosis, the glands show histological evidence of activity only during a brief period which begins on the second or third day after molting and ends around the middle of the stage.

5. The corpora allata are inactive on the day the grasshopper enters the adult stage, but the histological changes which lead to the production and release of secretory material begin immediately thereafter. Full secretory activity is achieved in the corpora allata of adult females shortly before the onset of the production of oviducal secretion and yolk. It is reached at an equivalent time in adult males.

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INFLUENCE OF GENETIC ENVIRONMENT ON THE REDUCTION OF BRISTLES BY THE DICHAETE GENE IN DROSOPHILA MELANOGASTER

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Introduction

The interaction and influence of one gene upon another is one of the methods employed in the study of gene action. The influence on the Dichaete gene can be readily measured, for this gene removes the bristles in the region of the presutural bristles. Thus, a bristle is either present or absent, and the quantitative effect can be accurately determined. Various mutations were brought into combination with the Dichaete gene, and the effect on the number of bristles was recorded.

Gene action has been analyzed by studying the effects of different dosages of mutant genes upon the phenotype (Stern, 1929, 1943; Schultz, 1935). Another method is to prolong larval life by means of low temperatures, genes or starvation (Green and Oliver, 1940; Green, 1946; Dunn and Coyne, 1935). High temperatures, which shorten larval life, cause a decrease in the number of bristles in the mutant Dichaete (Plunkett, 1926), and alter the phenotype in other Drosophila mutants (Stanley, 1931; Child, 1935; Harnly, 1936). Neel (1941) and Sparrow and Reed (1940) reported on the interaction of mutants that affect the chaetae of *D. melanogaster*. Stone (1947) used the effect of several mutants on Dichaete in an attempt to discover whether differences existed in reciprocal crosses.

An attempt was made to find a relationship, if one existed, between the number of bristles of the Dichaete mutant and the following mutations: Bar (B), black (b), Curly (Cy), eosin (w^e), eyeless (ey), heldout (ho), Lobe^2 (L^2), vestigial (vg), white (w), and yellow (y). The above mutants were picked for two reasons: one is that they were not in the third chromosomes, and that the mutations by themselves had no apparent effect on the thoracic bristles.

Methods

The Dichaete gene and the above mutant genes were placed in a common genetic background by the following method. The highly inbred Dichaete stock (inbred brother and sister for nine generations) was crossed to the mutants and then the mutants were extracted. This procedure was repeated eleven times for each mutant, and according to the formula developed by Bartlett and Haldane (1935) about 14 to 18 units on either side of the mutant gene remained heterozygous.

Plunkett (1926) reported that the bristle number of Dichaete flies was influenced by poor food conditions, high temperatures and crowding. Since the environment has a definite effect on bristle number, all matings were made under uniform conditions. The flies were raised at 24° C., and the same amount of

cornmeal-agar formula was used in each one-half pint bottle. Pair matings were used, and the parents were removed at the end of four days.

Bristles were counted on the right side of the scutellum, mesonotum, and on the right humerus. There are a total of twenty-six thoracic bristles, and the bristle count of the right half of the wild fly was then thirteen. The mean bristle count for the Dichaete stock was 10.634 ± 0.306 (♀♀) and 10.789 ± 0.147 (♂♂). The presutural, anterior supra-alars, and anterior dorsocentrals were the bristles that were usually missing in the Dichaete stock.

TABLE I

Genotype	Mean number of thoracic dorsal bristles of one-half of the fly (right side)			
	♀♀	No.	♂♂	No.
D/+	10.634 ± 0.306	592	10.789 ± 0.147	493
D/+ *B*/+	10.325 ± 0.896	623	10.394 ± 0.642	601
D/+ *b*/*b*	10.723 ± 0.711	246	10.641 ± 0.541	315
D/+ *Cy*/+	8.724 ± 0.742	419	8.970 ± 0.866	486
D/+ *ey*/*ey*	10.774 ± 0.419	283	10.861 ± 0.302	206
D/+ *ho*/*ho*	12.818 ± 0.149	336	12.444 ± 1.148	376
D/+ L^2/+	10.191 ± 1.391	484	10.889 ± 0.168	479
D/+ *vg*/*vg*	11.250 ± 0.521	229	12.253 ± 0.636	288
D/+ *w*/*w*	10.841 ± 0.413	396	10.718 ± 0.662	384
D/+ w^e/w^e	10.639 ± 0.523	344	10.392 ± 0.493	369
D/+ *y*/*y*	10.614 ± 0.614	248	10.743 ± 0.714	293

The Dichaete stock was crossed to each of the above mutants. Both ♀ and ♂ Dichaete flies were employed. The thoracic bristles were counted in the F_1 in the case of *B*, *Cy*, L^2 and in the Dichaete males containing *w*, w^e, and *y* genes. The other combinations were counted in the F_2.

RESULTS

In Table I the mean (or average) and the standard deviation of the mean of the thoracic dorsal bristles (right side) of *D*/+ and other genotypes are presented. In the Dichaete Curly cross, the *D*/+ *Cy*/+ flies had a lower mean (8.724 ± 0.742♀♀). This is a highly significant difference compared to mean of 10.634 ± 0.306♀♀ of *D*/+, for the difference is more than three times the probable error. The genes *vg* and *ho* significantly increased the mean of the bristle number to 11.250 ± 0.521 and 12.818 ± 0.149 respectively for females. The *B*, *b*, *ey*, L^2, *w*, w^e, and *y* genes had no influence upon the thoracic bristles when in combination with the *D* gene. The males had a slightly higher mean number of bristles than the females.

DISCUSSION

The action of the Dichaete gene had been postulated by Plunkett (1926) to remove bristles in a direction radiating outward from the presutural bristle. A catalyst which decomposes another bristle-forming catalyst was the manner in which bristles were supposed to be removed. Neel (1941) questioned the ex-

istence of the above diffusion hypothesis in scute, but in counting the bristles of Dichaete flies and Dichaete-mutant combinations it was evident that a gradient existed. In flies with 12 bristles on the right side, it was always the presutural bristle that was missing. In the *D*/+ *Cy*/+ combination, the bristles anterior notopleural, anterior supra-alars, anterior dorsocentral, and upper humerals were the bristles that were usually missing. There was a gradient present in the direction of the anterior supra-alar bristle. It is possible that the bristle-removing substance produced by the Dichaete gene is different from that found in scute, although they both remove bristles.

The Dichaete gene evidently produces substances directly or indirectly that destroy or inhibit the bristles. Now this system is disrupted by high temperatures which evidently speed up a reaction or reactions which produce more bristle-destroying or inhibiting substance, and thus a fly with fewer bristles is the result. The above system of removing or limiting the number of bristles is thrown out of balance by poor food conditions and low temperatures which evidently slow down the reactions and permit the bristle-destroying catalyst or bristle-inhibiting substance to act for a longer time, again producing flies with fewer bristles.

One encounters some difficulty in using the above scheme in explaining the effect of genes upon the Dichaete bristle system that neither increases nor decreases the life cycle of the flies. The *Cy* gene reduces the number of bristles in a Dichaete fly, and yet it neither speeds up nor slows down the length of time the individual insect spends in egg, larval, or pupal stages. Evidently the *Cy* gene produces in some way a larger amount of the bristle-destroying or inhibiting substances or may cause these substances to begin reacting earlier and to last longer.

The Curly gene turns the tips of the wings upward, and is a small inversion in the second chromosome. *D* is also a result of an inversion, and besides removing bristles also spreads the wings apart at a 45° angle. This may be a coincidence, or there may be a relationship between wing mechanism and bristle formation. The presence of genetic modifiers in the *Cy* inversion has to be considered. However, the method of inbreeding and selection would result in the *Cy* flies all having the same chromosome rearrangement. If the *Cy* flies used contained different genetic modifiers, the *D*/+ *Cy*/+ flies would have a greater standard deviation of the mean (would be more variable) than the *D*/+ flies. This difference is not significant (Table I).

The *vg* and *ho* genes are wing mutants, and they increase the length of time spent in the larval stage. One would expect a low bristle number according to Plunkett's hypothesis (1926), but that is not the case (Table I). It may be the interruption of the wing forming mechanism that influences the removal of bristles by the Dichaete bristle destroying substance. There appear to be several unrelated factors which can cause a variation in the number of bristles reduced by the Dichaete gene. Thus, the bristle destroying substance does not act simply upon a bristle forming substance. One cannot say that a substance *A* produces *B* which is a precursor of a bristle destroying substance *C* which acts upon a substance *D* which in turn produces bristles. There are probably more than three substances in the chain, and there is evidence, because of diverse environmental and genetic modifiers, that numerous side chain reactions exist that influence the step by step production of the bristle destroying or inhibiting substance. It is

a complex process in that these variable factors may work at different times or several together at various times. Temperature, food conditions, other seemingly unrelated genes, and chromosome arrangements play an important role in the action of the Dichaete gene.

Summary

A highly inbred Dichaete (*D*) stock of *D. melanogaster* was crossed to isogenic stocks carrying various mutations, which by themselves had no apparent effect upon thoracic bristles. The Curly gene (*Cy*) in combination with *D* decreased the average number of one half the thoracic bristles from 10.634 ± 0.306(♀♀) to 8.724 ± 0.742. The *Cy* gene is an inversion on the second chromosome, and it aids the *D* gene, which is on the third, in the removal of a larger number of bristles. Vestigial (*vg*) and heldout (*ho*) increase the bristle number to 11.520 ± 0.521 and 12.818 ± 0.149 respectively. *Vg* and *ho* increase the larval life, and disrupt the production of a wild type wing.

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STUDIES ON ARTHROPOD CUTICLE. II. ELECTRON MICROSCOPE STUDIES OF EXTRACTED CUTICLE[1, 2, 3]

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In previous work with the electron microscope it was found that sections of cockroach cuticle showed alternating denser and less dense laminae (Richards and Anderson, 1942a). Following treatment with hot alkali solutions this density differentiation was lost. Several possible explanations were suggested in the above paper, the two chief ones being that either a localized heavy component (e.g., protein) in the dense laminae was removed, or that the alkali treatment not only removes certain components but also causes or is followed by a redistribution of the remaining components (at least principally chitin). The x-ray diffraction studies of Fraenkel and Rudall (1940, 1947) suggest that the latter explanation is more probably correct. The present studies were undertaken to clarify this point further and to evaluate the use of isolated cuticles in permeability studies.

In general, we have found that any treatment leading towards chitin purification alters the membrane structure extensively. It follows that the extraction method of analysis has little value for a study on localization of components in the cuticle, that the cuticle cannot be viewed as a fixed framework of chitin micelles in which the other components are embedded, and that purified chitinous membranes, such as used by Yonge (1936) and Alexandrov (1935), are without biological significance for studies on the permeability of arthropod cuticle although normal isolated cuticles may give valid data. Unexpected variations between purified chitin membranes from different sources were found; the significance of these variations is discussed.

Materials and Methods

In our electron microscope studies on arthropod cuticle we have found it most satisfactory to perform the bulk of the experimental and observational work on readily prepared thin membranes. Subsequently results are checked by examination of other cuticular membranes and, when necessary, with sectioning and other ancillary methods. Accordingly the bulk of the work recorded in the present paper was done with large tracheae from a cockroach (*Periplaneta americana*). These tracheae contain chitin as well as protein, polyphenol and lipid components. Data obtained with cockroach tracheal membranes were then checked with crop linings,

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[2] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Minnesota. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

[3] Acknowledgment is due to the Electron Microscope Committee of the University of Minnesota for making the instrument available to us, and to Miss Virginia Kletzin for technical assistance.

rectal linings, soft general body cuticle, wing membranes, various types of scales and setae, and other tracheal membranes including ones consistently negative to chitin tests. The work recorded in the present paper was restricted to membranes sufficiently thin for direct electron micrography.

Normal structure was determined from membranes dissected out in saline solution, rinsed briefly in distilled water, and then placed on electron microscope screens and air-dried (Richards and Anderson, 1942 a, b, c; Richards and Korda, 1947, 1948). Tests on series of specimens treated with distilled water for periods ranging from several minutes to several hours at room temperatures showed no significant differences as far as can be detected with electron micrographs; if the brief rinse in distilled water necessary to clean the surface of the tracheal membranes has any effect on the membrane itself we were not able to detect it (see also under water extraction experiments in main section of this paper). The larval cuticle of mosquitoes and membranes from the gut were wiped gently with a soft brush to facilitate cleaning. The mosquito wing membranes were mounted directly without any treatment except the indicated experimental treatments. The membranes used in the present studies are reasonably large; accordingly they were placed directly onto the screens used in electron micrography (i.e., no supporting membrane of collodion, formvar, etc.) and the membrane under consideration is the only membrane in the field.

Experimentally treated membranes were handled in the same manner except for receiving the indicated treatments. In all experiments, a piece of membrane was removed from the animal, divided into two or more pieces, one of which was always mounted as control for the other piece or pieces which were given treatments. All tests recorded were run in duplicate, and many of them were repeated.

In electron microscope work on arthropod cuticle it is always necessary to consider the possibility of instrumental errors, especially the destructive effects of electron bombardment and of heat produced thereby (Richards and Anderson, 1942a; von Borries and Glaser, 1944). The more gross effects recorded in the present paper can be readily seen by dark-field microscopy or phase-contrast microscopy in membranes still in the solutions used. They are therefore not produced by either the drying or the electron bombardment—although it is quite possible that some of them may be augmented by these factors. The similar appearing but more minute effects cannot be observed directly with a light microscope but it seems reasonable to presume that those shown in the present paper are, like the gross effects, produced by the chemical treatment because birefringence values do not change significantly on drying.

As is generally known, one may remove from insect cuticle (a) the water, (b) the lipids, (c) the water-soluble protein, or (d) presumably all the components other than chitin.[4] No method is known for the removal of chitin without the simultaneous complete dissolution of the membrane. Since electron micrography must be performed in a vacuum, it has not been possible to study hydrated membranes. Treatments used were selected from the literature, and were chosen to cover the various methods that have been used for the removal of certain components, especially those used for the purification of chitin.

[4] Calcium salts which make up so large a part of many crustacean cuticles are absent from almost all insect cuticles (absent from all of those studied by us).

Table of Treatments Employed

Agent	Strength	Temp. (°C.)	Time	Type of cuticle
KOH	5%	20	6 + 10 days	C.T.
	5%	100*	1 day	C.T., M.W.
	20%	25	2 days	M.L.C.
	20%	65	2 days	M.L.C.
	20%	85	4 hours	C.T.
	20%	100*	1 day	M.W.
	40%	100*	10 minutes	L.P.M.
	conc. (= ca. 60%)	160*	15 min. to 2 hrs.	C.T., C.H.G., C.C., Ce.S., L.C., M.W., M.L.C., (H.B.A.S.)†
NaOH	5%	20	2–12 days	C.T., M.L.C., H.C., M.L.T., Ce.S.
	5%	65	6 + 10 days	C.T.
	5%	100*	5 + 9 days	C.T.
	10%	100*	1 day	(H.B.A.S.)
	20%	85	4 hours	C.T.
HCl	5%	100*	1 day	C.T.
Pepsin (+ HCl)	10%	36	1 day	C.T., L.C., L.P.M., H.B.A.S.
Diaphanol‡	full	25	1, 5, 10 + 41 weeks	C.T., C.C., (H.B.A.S.)
$KMnO_4$	0.1%	20	2 days	C.T.
H_2O_2	30%	20	2 days	C.T.
$CHCl_3$ (through acetone)	C.P.	reflux	5 min. to 2 hrs.	C.T., H.B.A.S.
$CHCl_3$ then pepsin	as above	as above	$CHCl_3$ for 2 hrs. pepsin for 1 day	C.T., H.B.A.S.
Aerosol OT§	0.02%	25	1 day	H.B.A.S.
Isotonic salt sol.	—	25	2–7 days	C.T., H.B.A.S.
Distilled water	—	25	few min. to 2 weeks	C.T., H.B.A.S., M.L.C.
	—	30	1 + 2 days	H.B.A.S., M.W.
	—	60	1, 7 + 14 days	C.T., M.W.
	—	65	1, 2 + 7 days	H.B.A.S., M.L.C.
	—	100*	1, 7, 14 + 28 days	C.T., H.B.A.S., M.W., M.L.C.
	—	reflux	5 days	C.T.

* In sealed glass ampoules.

† Parentheses indicate that the membrane was completely destroyed by the treatment.

‡ Saturated solution of chlorine dioxide in 50 per cent acetic acid.

§ Di octyl sodium sulfo succinate.

C.C. = Cockroach (*Periplaneta americana*), membrane lining crop.

C.H.G. = Cockroach, membrane lining hind gut.

C.T. = Cockroach, trachea (large trachea extending anterior from prothoracic spiracle to head).

Ce.S. = Centipede, sensillae (Richards and Korda, 1947).

H.B.A.S. = Honey bee, abdominal air sac (parentheses indicate destroyed).

H.C. = Housefly, membrane lining crop.

L.V. = Blowfly (*Lucilia illustris*), membrane lining crop.

L.P.M. = *Lucilia*, peritrophic membrane.

M.L.C. = Mosquito (*Aedes aegypti*), larval cuticle of abdomen.

M.L.T. = Mosquito, larval tracheae.

M.W. = Mosquito, wing membrane.

The preparations given lengthy treatments and those treated at higher temperatures were placed in sealed glass ampoules. Most of the hydroxide treatments were given in pyrex glass but a few were run in tubes of fused silica. The preparations from membranes treated with alkali must usually contain a trace of alkali because this is not completely washed out of cuticle by rinsing in water (or even prolonged soaking). Probably more alkali remains in those preparations washed in alcohol. Full removal of the alkali by soaking in one per cent HCl followed by washing in distilled water does not alter the electron microscope picture; accordingly we have usually ignored this trace of alkali that may remain in the preparation (Figs. 5–6 and 10–12 show preparations treated with HCl to remove all the alkali, while Figs. 7–9 were not washed in acid solution). The effects of washing in various ways is discussed in the next section. Diaphanol is an oxidizing agent prepared by saturating a 50 per cent solution of acetic acid with sulfur dioxide; it can be washed from cuticle with water but we used the supposedly gentler treatment (recommended by Koch, 1932) of transferring through 50 per cent, 30 per cent and 10 per cent acetic acid to water, then washing in one per cent $NaHSO_3$ at room temperature or 2 per cent $Na_2S_2O_3$ at 97° C., and finally washing again in water. Preparations treated with potassium permanganate were washed first in water, then in 0.1 per cent $NaHSO_3$, and then again in water. Specimens refluxed with chloroform were transferred from water to chloroform through acetone, and back to water through acetone; they were dried from water. For extraction with aqueous media, double distilled water was used, the second distillation being made in a pyrex glass still.

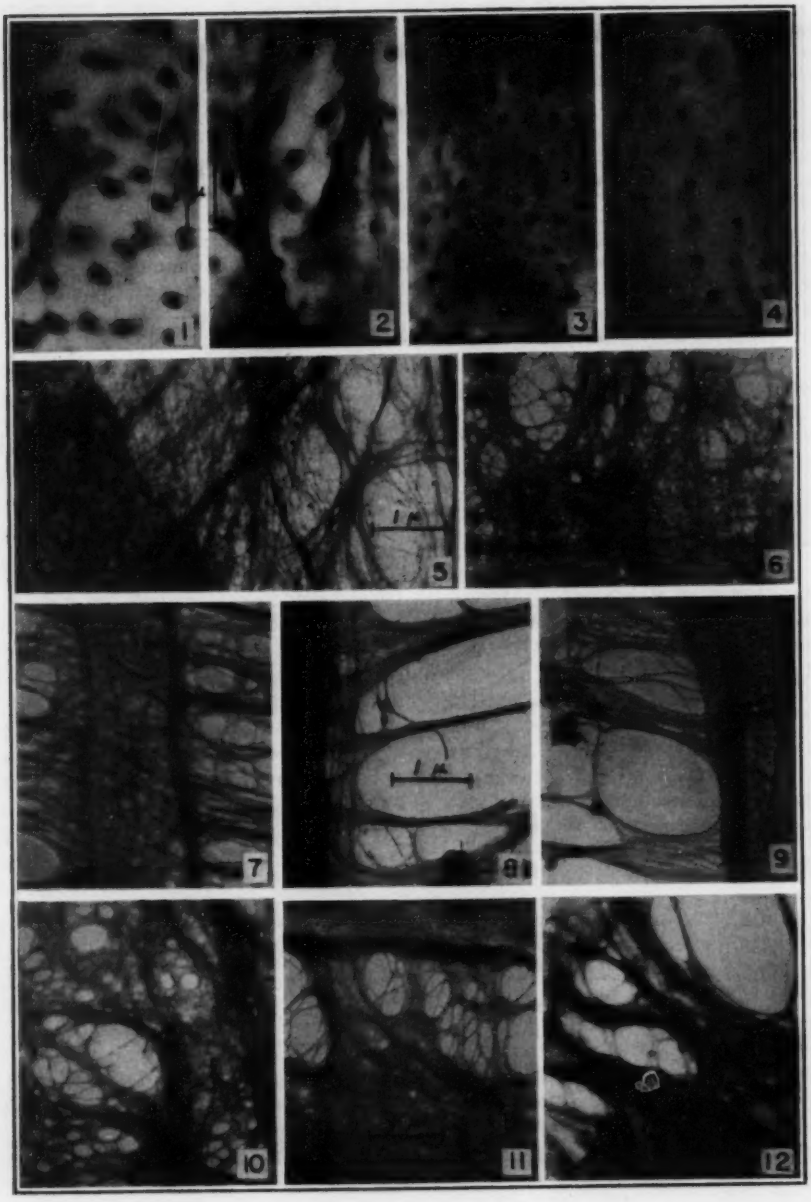
The electron microscope used was an RCA model EMU which uses an accelerating potential of 50 kilovolts. Most of the present work was done with the standard instrument as received from the factory. More recent pictures (those bearing numbers above 300) were taken after modifying the instrument by installing a saturation emission type electron gun and compensating the objective lens and after introducing the recently developed operational refinements to increase resolution (Hillier and Ramberg, 1947). Electron micrographs were made at an initial magnification of 6,000–10,000 and photographically enlarged to the size used for reproduction. Resolution in most of the figures of thin membranes presented is better than 0.01 μ; in some of the best it is of the magnitude of 0.005 μ; resolution in thicker membranes (wings, mosquito cuticle) is less good (probably 0.02–0.05 μ).

The Normal Structure of Arthropod Cuticle

Only a cursory summary to provide background for the following sections will be given. In current terminology the cuticle is divided into two major subdivisions distinguished by the presence and absence of the polysaccharide chitin. The outer layers of the cuticle which contain no chitin are termed "epicuticle," and the inner layers composed of a laminated chitin and protein matrix are termed "endocuticle." The epicuticle is usually distinctly double, consisting of an outer "lipid epicuticle" and an inner "protein epicuticle" (Richards and Anderson, 1942a; Dennell, 1946; Wigglesworth, 1947). Numerous further subdivisions are commonly recognizable but the above three will suffice for the purposes of the present paper.

The above three subdivisions are not necessarily all present. The cockroach has all three subdivisions in the thick cuticle covering the outside of its body and

PLATE I



also in the very thin cuticle covering its crop and its tracheae (despite the fact that these membranes are only in the range of 0.01 to 0.1 μ thick when dry). The same is true for wing membranes. Using the above terminology, the air sac and tracheae of honey bees consist only of epicuticle but have both lipid and protein subdivisions of this. The abdominal cuticle of mosquito larvae lacks a demonstrable lipid layer; it may be said to consist of a protein epicuticle plus an endocuticle. The peritrophic membrane (in the midgut) is a special case due to its peculiar origin; it contains both protein and chitin (Wigglesworth, 1939). For further analysis see papers cited in the bibliography.

Trachea are tubular invaginations of the body wall. In keeping with their origin they possess a secreted cuticle which lines the inner surface of the tubes. This cuticle is continuous with the cuticle of the external surface of the body, and the two are generally considered to be of homologous composition. These thin cuticular tubes are made more rigid by the development of supporting helical thickenings in the endocuticle. The helical thickenings are called taenidia. In large tracheae the taenidia are rather thick—too thick for adequate penetration by a 50 kV electron beam—but the membrane between the taenidia is usually quite thin (after drying ranges between 0.01 and 0.05 μ thick in species treated herein) and can be readily examined with an electron microscope (Richards and Anderson, 1942 b, c). Commonly, after removal of part of the material the taenidia decrease in density sufficiently for adequate electron penetration (Figs. 7, 17, etc.).

The intertaenidial membrane is seldom of uniform composition (Richards and

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PLATE I

Cockroach (*Periplaneta americana*), large tracheae from prothorax

FIGURE 1. Normal; intertaenidial membrane. The spots are thickenings in the endocuticle. (No. 126e) 16,000 ×.

FIGURE 2. Normal; intertaenidial membrane with edge of taenidium at lower margin; bars are braces extending from taenidium onto membrane. (No. 121d) 16,000 ×.

FIGURE 3. Normal; intertaenidial membrane with edges of taenidia at upper and lower margins. (No. 66c) 12,000 ×.

FIGURE 4. Washed with acetone, then refluxed with boiling chloroform for 5 minutes. (No. 178d) 16,000 ×.

FIGURE 5. Treated with 5 per cent KOH at 20° C. for 6 days, washed in 1 per cent HCl, then water. At left is broken, partially disintegrated epicuticle, at right only endocuticle. (No. 48c) 12,000 ×.

FIGURE 6. Treated with 20 per cent KOH at 85° C. for 4 hours, washed in 1 per cent HCl, then water. Only endocuticle of membrane. (No. 66d) 12,000 ×.

FIGURE 7. Treated with conc. KOH at 160° C. for several hours, washed in water. Taenidium vertically through center of picture, intertaenidial membrane on both sides. Resolution relatively poor. (No. 14c) 12,000 ×.

FIGURE 8. Treated with conc. KOH at 160° C. for 20 minutes, washed in water. Example with larger holes. (No. 19a) 12,000 ×.

FIGURE 9. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in alcohol. Taenidium placed vertically on right, membrane on left. (No. 198c) 12,000 ×.

FIGURE 10. Treated with 5 per cent NaOH at 20° C. for 10 days, washed in 1 per cent HCl, then water. Intertaenidial membrane only, with partially disintegrated brace extending from lower center upwards. (No. 46b) 12,000 ×.

FIGURE 11. Treated with 5 per cent NaOH at 65° C. for 6 days, washed in 1 per cent HCl, then water. Intertaenidial membrane only. (No. 32c) 12,000 ×.

FIGURE 12. Treated with 20 per cent NaOH at 85° C. for 4 hours, washed in 1 per cent HCl, then water. Intertaenidial membrane only. (No. 59e) 12,000 ×.

Korda, 1948). It usually either is studded with thickenings in the endocuticle (Figs. 1–4) or contains a reticulate meshwork of thickenings (Figs. 45–56). The structural details revealed in electron micrographs of tracheal membranes show more variability than one would like for precise quantitative work, but the changes discussed are so great in comparison to this variability that we may, as far as the present paper is concerned, treat the normal membrane structure as though constant.

Effects of Lipid Extraction

The lipid epicuticle is not ordinarily detectable in mounts of entire membranes because it is so delicate (and seemingly so uniform) that it is lost against the stronger background of protein and chitin-protein layers. To demonstrate it convincingly with an electron microscope one has to isolate the layer (Richards and Anderson, 1942a). The figures given in the present paper show only the protein epicuticle and the endocuticle even when the lipid epicuticle is still present.

As can be seen from Figures 4 and 48, treatment with acetone and boiling chloroform produces no distinct changes in either the protein epicuticle or the endocuticle. Seemingly the effect of lipid solvents is indeed limited to removal of the superficial lipid layer, as Wigglesworth (1945) and Beament (1945) assumed.

Effects of Chitin Purification

Extensive studies were made on the effects of chitin purification by various methods with the large tracheae from the prothorax of the cockroach as standard test material (see table). The chitin pictures obtained with this particular material were strikingly similar irrespective of the chemical method used for purification, irrespective of whether the purification was partial or what is referred to in the literature as complete, and irrespective of whether the chitin molecules were left unchanged or converted into chitosan (Figs. 5–23).

Figure 5 represents an incomplete purification with alkali. At least it is incomplete in the sense that the protein epicuticle is not completely destroyed; the epicuticle is clearly discernible in somewhat altered but recognizable condition over the left-hand part of the figure. Since it is not feasible to strip all the epicuticle off manually and test the endocuticle for proteins it is not possible to say whether the fibrous meshwork is pure chitin or still contains some protein. Ninhydrin and xanthoproteic tests on such membranes are positive but this might be due solely to the only partly disintegrated protein epicuticle. Figures 9 and 10 are other presumably incomplete chitin purifications with alkali, while Figures 6, 11 and 12 are supposedly complete chitin purifications. Figures 7 and 8 represent membranes in which the chitin has been changed into the de-acetylated form, chitosan. This picture is not significantly changed by staining with I + KI with or without a subsequent rinsing in one per cent H_2SO_4 (chitosan color test) or by prolonged soaking in a solution of potassium iodide. Figures 13–18 and 20 represent unknown degrees of purification with diaphanol, hydrogen peroxide, potassium permanganate, hydrochloric acid and pepsin. Figures 14 and 15 are probably pure or nearly pure chitin, the other probably only partially purified.[5] A

[5] We have experienced difficulty in obtaining negative ninhydrin tests on these membranes, even ones which considering the treatment should be highly purified chitin. Commonly we obtained negative xanthoproteic and Millon tests and yet a moderate positive ninhydrin test.

more obscure fibrous pattern is shown by the endocuticle in Figure 23 where the treatment with hot water can scarcely have given more than a partial purification to judge from comparison with the data of Fraenkel and Rudall (1940).

In all of these cases, whatever the degree of purification, the endocuticle of cockroach tracheae became primarily a fibrous meshwork (except for a delicate basal sheet to be discussed later). This is clearly not its normal state. Pictures of tracheae which have been deliberately abraded show areas where the epicuticle has been manually torn from the underlying endocuticle; in these cases the endocuticle appears as a true sheet without any discernible fibrous structure (Fig. 21). Also, so gross a fibrous structure would be discernible through the epicuticle in the control preparations (as it is in Fig. 23). Accordingly, we must conclude that the fibrous mesh is produced by the treatment.

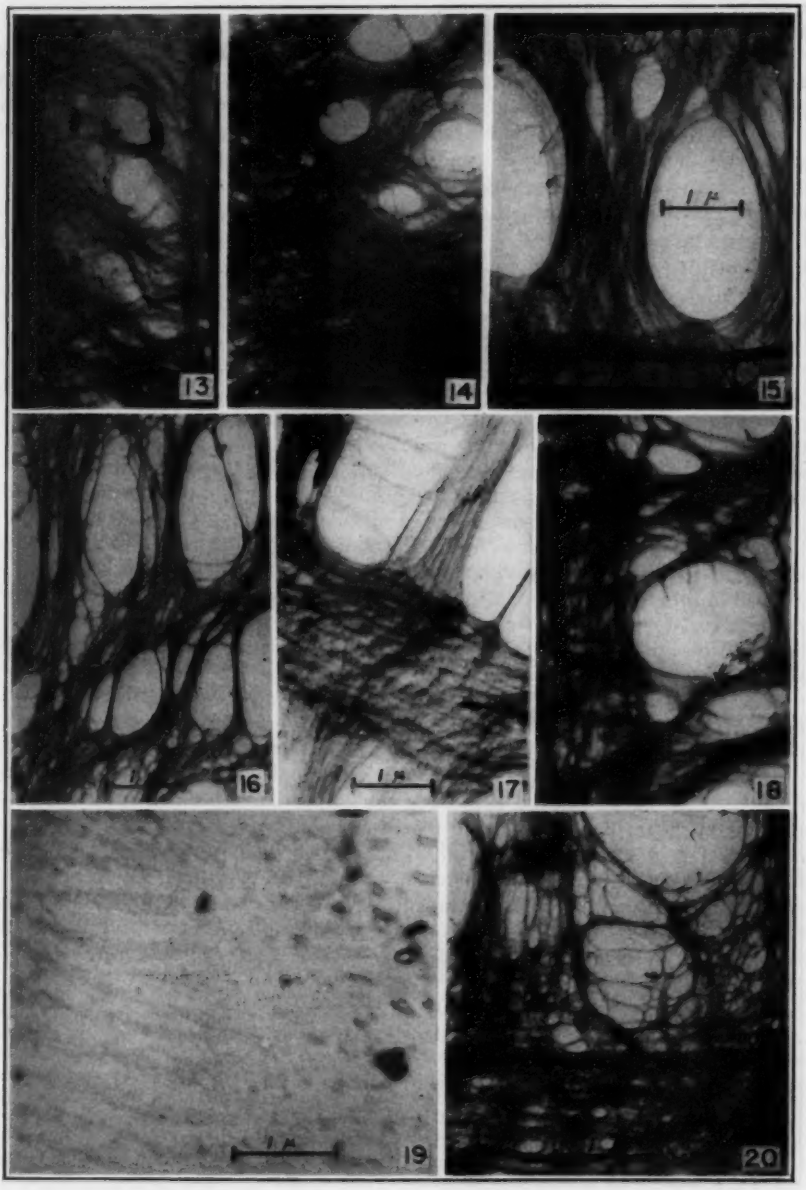
As can be seen from the pictures given, larger fibers can usually be resolved as multiples of microfibers. The microfibers of chitin are not of constant size, but variation among them does fall within a fairly narrow range in both tracheae and other membranes. Most of the microfibers have diameters in the range of 0.01 to 0.03 μ (100–300 Å) when dry. A few less than 100 Å in diameter were estimated as approximately 75 Å. The fact that in other membranes (see below) microfibers of similar diameters may be oriented randomly suggests that there may be some real significance to these dimensions. Perhaps the microfiber diameters ($< 100 - 300$ Å) represent the range of micelle or crystallite dimensions (the a and c axes thereof) but we have no proof of this. The report by Clark and Smith (1936) of the preparation of a "micellar solution" of chitin makes more plausible the suggestion that micelles might orient into microfibers of micellar diameters on removal of other membrane components. The fact that the diameters (1 μ) of the natural fibrils teased from lobster tendons by Clark and Smith are many times larger than our chemically prepared microfibers does not invalidate the above suggestion.

The chitin microfibers do not make a constant pattern except that in general they run longitudinally in the taenidia and at a right angle to this in the membrane between taenidia (Figs. 7–9, 17, 18, 20). Usually a finer reticulation is produced from gentler alkali treatment (lower concentrations and lower temperatures).[6] What seems more significant in this respect is that the pattern developed depends to some extent on the method used to remove the alkali. This effect of washing was studied in greater detail with other membranes (see below) but the generality that more destruction and coarser structure results from washing in alcohol can be seen from comparison of Figures 9 and 10. It does not seem likely, therefore, that the chitin molecules are arranged as shown in Figures 5–20 in the normal tracheal endocuticle. It seems more reasonable to assume that the chitin molecules are

It is well known that ammonia and certain amines interfere with the ninhydrin test. Dr. W. M. Sandstrom of the Chemistry Department tells us that it would not be surprising if chitin gave a positive ninhydrin test, especially if de-acetylated. We have had to conclude that the ninhydrin test is not reliable for determining the removal of protein from chitin. Unfortunately most of our preparations were made and tested before we were aware of this, and accordingly we do not have as accurate information as we would like on the membranes used for electron microscope examination. However, subsequent tests using the same procedures indicated that the remarks in the text about purification are reasonably accurate.

[6] This is not apparent from the figures because mostly micrographs showing finer fibers and finer holes were chosen as illustrations.

PLATE II



arranged otherwise in the intact endocuticle, that they are held in place by the protein molecules, and that on removal of the protein they reorient themselves into these micro fibers which run parallel to the surface of the membrane (both before and after drying). This latter interpretation is in fair agreement with the x-ray diffraction data of Clark and Smith (1936) and Fraenkel and Rudall (1940).

Some explanation is required, however, for the consistent tendency mentioned above for the microfibers to run longitudinally in the taenidia and at a right angle to this between taenidia. The fibers in the intertaenidial membrane appear to cross the taenidia (Fig. 17), and actually a continuous fibrous membrane can be separated from the taenidia after drastic alkali treatment. It seems therefore that there is a continuous endocuticle with fibers of one orientation and attached to this the taenidia with fibers set at a right angle to those in the membrane. Both sets are parallel to the surface of the membrane, and both are distinct from the more delicate homogeneous sheet to be discussed in the next paragraph. Since chitin is an anisotropic substance it is possible to determine that there is a corresponding difference in the orientation of the chitin micelles. Using a polarized-light microscope and an appropriate compensator we have shown that when a trachea is oriented at 45° to the crossed Nicols, the taenidia are in the retarding orientation when the intertaenidial membranes are in the accelerating orientation, and vice versa. This is true for normal tracheae as well as for ones treated with alkali to purify the chitin or convert it to chitosan (amplitude of birefringence much greater after alkali treatment). It follows from this that the chitin micelles in the intertaenidial membranes are indeed at right angles to those in the taenidia.[7] This point, which is of little importance to the present discussion, will be treated further in a subsequent paper devoted to the structure of tracheal cuticles (Richards and Korda, 1948).

[7] Picken, Pryor and Swann (1947) record a mosaic of fibers for the chitin fraction of *Donacia* cocoons. While the details remain to be proven it is simple enough to rationalize such differences between tracheal and cocoon membranes on the basis of origin.

PLATE II

Cockroach (*Periplaneta americana*), large tracheae from prothorax

FIGURE 13. Treated with diaphanol at 25° C. for 1 week. Intertaenidial membrane; edges of taenidia at sides. (No. 50c) 12,000 ×.

FIGURE 14. Treated with diaphanol at 25° C. for 5 weeks. Taenidium and intertaenidial membrane. (No. 61a) 12,000 ×.

FIGURE 15. Treated with diaphanol at 25° C. for 5 weeks. Intertaenidial membrane with edges of taenidia at top and bottom. (No. 60a) 12,000 ×.

FIGURE 16. Treated with 30 per cent hydrogen peroxide at 20° C. for 2 days, washed in water. Intertaenidial membrane only. (No. 43b) 12,000 ×.

FIGURE 17. Treated with 0.1 per cent potassium permanganate at 20° C. for 2 days. Taenidium and intertaenidial membrane. (No. 41e) 12,000 ×.

FIGURE 18. Treated with 5 per cent HCl at 100° C. for 1 day (in sealed glass ampoule), washed in water. Taenidium on left, membrane on right. (No. 63c) 12,000 ×.

FIGURE 19. Epicuticle manually separated after treatment of trachea with 10 per cent pepsin in acid solution at 36° C. for 1 day, washed in water. Note differences in structure corresponding to taenidial and intertaenidial regions (see Fig. 20). (No. 58c) 16,000 ×.

FIGURE 20. Endocuticle manually separated after treatment of trachea with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. Taenidium at bottom, intertaenidial membrane above. (No. 58e) 16,000 ×.

In addition to the two sets of fibrous sheets treated in the preceding paragraph there is a continuous sheet in these purified tracheae. This continuous sheet is so thin and, more to the point, so homogeneous that it was overlooked by us for a long time. It is not discernable in any of the pictures reproduced here although it is present in Figures 8, 9 and some others. It is discernable in the occasional cases when it breaks after long bombardment. Once its presence was suspected it was readily demonstrated by placing small particles on it (e.g., a deposit of magnesium oxide smoke). This homogeneous sheet does not seem to be any part of the epicuticle because after separation of epicuticle from endocuticle by pepsin digestion it is found intimately associated with the chitin microfibers in the endocuticle. Likewise it does not seem possible for it to be the basement membrane of the tracheal epithelium because of alkali resistance, different appearance from known preparations of the basement membrane and intimate association with the fibrous endocuticle. It is not present in all preparations but we interpret this as probably meaning that it is commonly lost in the course of preparation. We know only three things about this membrane: its resistance to pepsin and hot alkali, its homogeneity (at least when dry) even in pictures where the resolution is to better than 50 Å (0.005 μ), and its intimate association with microfibers of the intertaenidial endocuticle. It is certainly a very thin membrane (probably less than 100 Å thick when dry) but we do not have pictures which permit an estimation of its thickness. Perhaps it is a sheet of chitin but one would have to obtain this sheet free from the known chitinous fibers before reliance could be placed on the chitin color tests. Further work is needed on the nature of this sheet.

The protein epicuticle is destroyed by the various treatments used. However, if the treatment is slight enough to give only partial purification, a reasonably normal protein epicuticle may be peeled off the modified endocuticle. This is shown particularly well by Figure 19 (which was stripped from the preparation that gave Figure 20). This epicuticle (and also the bee air sac) never shows a fibrous structure such as the endocuticle of cockroach tracheae does. In the early stages of purification the endocuticle is more readily altered than is the protein epicuticle. This suggests that the protein is more easily removed from the chitin-protein binding than from membranes where protein chains are linked directly to one another. Otherwise stated, it would seem that, if we assume only one protein species is involved, the cuticular proteins of cockroach tracheae are less strongly bound when linked to chitin than when linked to other protein molecules.

Similar but less extensive studies were made with a number of other types of cuticle. In single tests similar results were obtained with tracheae of *Blatta orientalis* and *Galleria mellonella*, but tracheae of *Neodiprion lecontei* became only vaguely fibrous, and those of *Calandra oryzae* retained their normal appearance. The cuticle lining the crop (part of fore gut) of adult flies gave results essentially similar to those from roach tracheae.[8] On treatment with pepsin the crop membrane of a blowfly developed numerous small holes and a vague fibrous structure (Fig. 28). On treatment with hot concentrated alkali it developed a heavy fibrous mat in which the relative vagueness of the individual fibers is at least partly due

[8] Considerable difficulty is encountered in getting gut membranes sufficiently clean for electron microscopy. Controls are only seldom clean enough to permit comparison with treated preparations, and then usually only clean in spots. Blurred areas on Figures 27 and 30 are interpreted as adhering debris.

to the decreased resolution occasioned by thickness of the preparation (Fig. 29). It seems that the crop membrane differs from the cockroach tracheae principally in having the chitin fibers oriented randomly and in requiring somewhat more drastic chemical treatment for purification. The crop lining of the closely related housefly gave similar results: mild alkali treatment gave relatively little change and only a very vague fibrous appearance when the alkali was removed with water (Fig. 31); removing the alkali with alcohol did not increase the fibrous appearance but, as usual (see below), resulted in a less normal appearing membrane with numerous minute holes (Fig. 32).

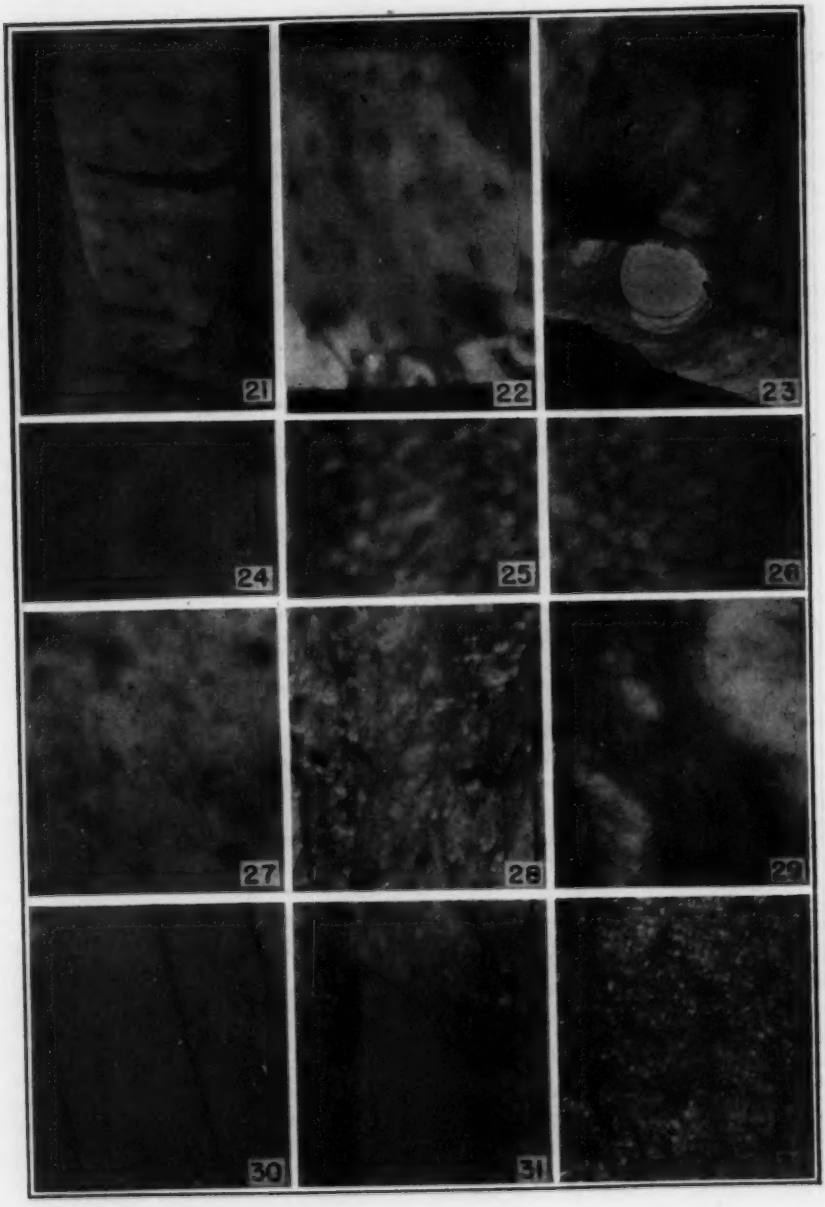
The heavier membrane lining the crop of the cockroach is more resistant. Ignoring the heavy, spine-like projections and considering only the homogeneous membrane of the controls (Fig. 33), we find an irregular removal of material by hot concentrated alkali (Fig. 34). This alkali treatment was quite drastic and is commonly thought to remove all components other than chitin and also to convert all the chitin to chitosan. Yet the membrane is still intact and shows islands of greater density. Prolonged treatment with diaphonal (41 weeks) gave a different picture in which a vague, unoriented fibrous structure is apparent, with minute holes visible in the thinner patts (Fig. 35).

The peritrophic membrane of the midgut of adult flies gave pictures not encountered with any other membrane examined. This membrane has a different origin from ordinary cuticle but agrees with endocuticle in being composed of chitin and protein (Wigglesworth, 1939). Sufficiently clean controls show a seemingly homogeneous membrane (Fig. 24). Treatment with pepsin and alkali gives the same altered picture (Figs. 25–26); an intact membrane with relatively gross, denser reticulations which presumably represent reticulate thickenings (reminiscent of the reticulations seen in the normal intertaenidial membranes of many tracheae, compare Figure 55).

Wing membranes of adult mosquitoes showed the least alteration of any illustrated in this paper. Mild alkali treatment gave no detectable change (Fig. 36). Drastic alkali treatment resulted in the production of some vesicles (Fig. 37) or actual holes (Fig. 38). None of the treatments revealed any fibrous meshwork. Equally great resistance to alkali treatment was shown by centipede sensillae (Richards and Korda, 1947) and by strongly "chitinized" butterfly scales (unpublished), and by the large tracheae of the beetle, *Calandra oryzae*. These showed only a considerable decrease in density.

The cuticle of mosquito larvae has already been fractionated and studied (Richards and Anderson, 1942a). Some of these experiments were repeated especially to study the effects of various types of washing following alkali treatment. In preparing alkali-treated membranes for permeability studies Yonge (1936) washed in 95 per cent ethyl alcohol and then transferred to water for permeability tests. He assumed (erroneously) that the alkali treatment removed only the epicuticle but did not affect the endocuticle. Normal structure of mosquito larval cuticle is shown in Figure 39; it shows a fairly uniform membrane crossed by smooth parallel ridges approximately one micron apart. After an alkali treatment that should leave pure or nearly pure chitin, diverse pictures are obtained depending on the washing procedure. Washing in water results in a membrane with numerous, rather crowded, lighter spots that seem to represent vesicles in the membrane (Fig. 42); one could interpret this as representing randomly oriented vague

PLATE III



chitin microfibers. If the treated membrane is washed in alcohol and then in water, a vague but distinct fibrous appearance is obtained (Fig. 40). If the treated membrane is washed only in alcohol many holes but no distinct fibers result (Fig. 41). If the treated membrane is washed in water first and then in alcohol a more nearly uniform membrane results with only a hazy suggestion of fibrous structure (Figs. 43–44). From this series of experiments, as well as from less extensive experiments with different washing procedures on other membranes (as noted in preceding paragraphs), it is obvious that the structure of the treated membranes is affected not only by the alkali but also by the subsequent treatment. The chitin molecules must be labile indeed for a water treatment to return a membrane such as that shown in Figure 41 to the condition of that shown in Figure 40. It does not seem desirable to attempt interpreting the diverse set of pictures shown in Figures 40–44 other than to say that clearly no one of them is "normal," that no one would have any biological significance if used in a permeability experiment and that washing alkali out with alcohol seems to cause more deviation from normal membrane structure than washing with water does.

Effects of Extraction with Water and Aqueous Solvents

Fraenkel and Rudall (1940, 1947) and others have shown that some of the protein of insect cuticles is extractable with hot water. There is an inverse relationship to sclerotization, i.e., soft, non-sclerotized cuticles lose much of their protein on extraction with hot water, while hard, sclerotized cuticles lose relatively little.

Plate III

All figures reproduced at 16,000 ×

Figure 21. Cockroach trachea. A normal trachea manually abraded. The epicuticle has been torn and peeled away from a small area leaving the intact endocuticle exposed. (No. 384c)

Figure 22. Cockroach trachea. Treated with distilled water at 100° C. for one week. (No. 336e)

Figure 23. Cockroach trachea. Treated with distilled water at 100° C. for 2 weeks. Area of approximately maximum effect. (No. 340d)

Figure 24. Blowfly (*Lucilia illustris*), peritrophic membrane. Normal, washed in water but probably not entirely clean. (No. 89e)

Figure 25. Blowfly, peritrophic membrane. Treated with 40 per cent KOH at 100° C. for 10 minutes, washed in water. (No. 87b)

Figure 26. Blowfly, peritrophic membrane. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 78b)

Figure 27. Blowfly, crop lining. Normal, washed in water but not entirely clean. (No. 79b)

Figure 28. Blowfly, crop lining. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 80c)

Figure 29. Blowfly, crop lining. Treated with conc. KOH at 160° C. for 15 minutes, washed in water. (No. 79d)

Figure 30. Housefly (*Musca domestica*), crop lining. Normal, washed in water but speckles may be debris. (No. 193c)

Figure 31. Housefly, crop lining. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in water only. (No. 194a)

Figure 32. Housefly, crop lining. Treated with 5 per cent NaOH at 20° C. for 9 days, washed in 95 per cent ethyl alcohol. (No. 195a)

Since isolated cuticles are sometimes used in permeability studies, in our laboratory and elsewhere, it seemed desirable to see what we could learn from electron micrographs of membranes extracted with water and salt solutions at various temperatures.

Cockroach trachea soaked in distilled water at 25° C. for periods up to two weeks appeared normal. Ones soaked at 60° C. for one and two week periods seemed to be somewhat affected but the differences from control preparations were not well marked. Ones soaked at 100° C. showed a clear loss of substance, development of a network type endocuticle with some fibrillar structure, and a considerable tendency for the epicuticle to break (Figs. 22–23). Cockroach tracheae exhibit so much variability that they are useful only when the effects are striking (as in chitin purifications) and accordingly it is not safe to draw conclusions from the negative results of extraction with water at low temperatures.

The air-sacs of honey bees gave more clearly defined changes and so were studied rather intensively.[9] As far as can be ascertained from structure visible in the electron micrographs, soaking these membranes in distilled water at 25° and 30° C. for periods up to a week produces no change. At the higher temperatures which Fraenkel & Rudall used for extracting proteins from cuticles, the effects are striking. Air-sacs treated in distilled water at 65° C. for two days show obvious swelling of the ridges accompanied by decrease in sharpness and density (Fig. 49). Electron micrographs of air sacs treated with hot water do not always give this particular type of picture but do always contrast with control preparations. Another type of picture is shown by Figure 50, of a specimen soaked at 65° C. for a week; in this case the removal of material and the change has not been uniform over the preparation—as can be seen by comparison of right and left halves of this picture. Treatment with distilled water at 100° C. gives still greater change. After one day there is a general fuzziness suggesting removal of material from the ridges (Fig. 51). After eight days the membrane is much more delicate, the ridges and reticulations have mostly disappeared and the membrane between ridges is less dense and therefore thinner (Fig. 52). After a month soaking at 100° only a very delicate, but still continuous, membrane remains (Fig. 53).[10] The delicate membrane remaining in Figure 53 does not show properties of the lipid epicuticle, i.e., it does not disintegrate under electron bombardment; accordingly it may be presumed to represent one of the protein layers. These hot water extractions of air sacs show, as one might have predicted from the work of Beament (1946) and Wigglesworth (1947), that the protein is more readily removed from some portions than from others; seemingly protein is removed most readily from the thickenings and less readily from some of the continuous layers than from others. Whether there are as many discrete layers in the bee air sac as in the thicker epicuticles studied by Beament and Wigglesworth is not known but analysis of these extraction pictures suggests that more than one protein layer is present. This is also suggested, but not proven, by the membranes remaining after pepsin digestion (Figs. 54–55).

The resolution shown in electron micrographs of bee air sacs is excellent.

[9] One should remember that the air-sacs of honey bees are consistently negative to chitin tests.

[10] For focusing electron micrographs some contrast is needed. With such homogeneous membranes this is most readily accomplished, as was done here, by finding an area where minute particles of debris are present.

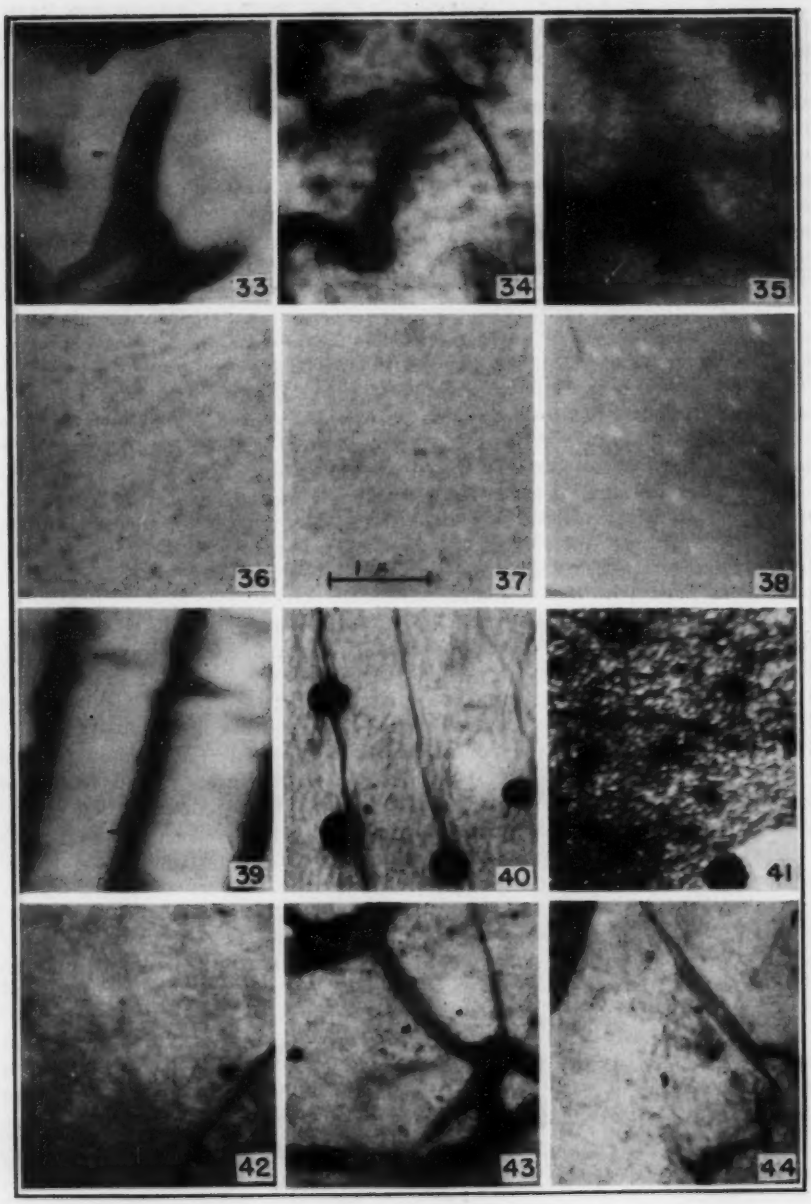
Sharp discontinuities of the diameter of 100 Å should be clearly visible, and in the best pictures sharply contrasting discontinuities (e.g., holes) of half this size should be detectable. However, since we are dealing with a multiple membrane where effects in one layer may be partially hidden by an overlying layer we cannot claim to have proven the absence of effects of this magnitude. To be on the conservative side we will only say that there seem to be no changes down to the range of several hundred Ångstrom units. One could therefore claim that there is good evidence that these air sacs could be used as isolated membranes for permeability studies at room temperatures. At high temperatures they are altered but at room temperatures even rather lengthy treatments give either only exceedingly slight alterations (beyond detection by the present electron micrographs) or else slight effects which are completely masked by drying.

Soaking honey bee air sacs in physiological saline solutions for several days at 25° C. likewise seems to cause no detectable effects (Fig. 48). The same may be said for dilute solutions of some single salts (e.g., KCl). However, it seems likely that strongly hypertonic salt solutions do affect these membranes deleteriously. The effects of hypertonic saline solutions are only slight; an adequately extensive analysis of them has not been completed. We can safely record, however, that strong aqueous solutions of agents commonly used to facilitate protein extraction can affect the structure seen in electron micrographs of bee air sacs (e.g., 6M urea, 5 per cent sodium salicylate, detergents). A number of detergents (anionic, cationic and non-ionic) have been tested in a preliminary manner. Different detergents do not give the same degree of effect but even in dilute solutions they show a considerable disruptive effect (Fig. 56). This fact is of some interest because Wigglesworth (1945), in studying cuticle permeability, compares the effect of removing the lipid epicuticle by the use of lipid solvents with the presumed disruption of the lipid epicuticle by the use of detergents. Clearly this comparison is not a simple one because we can demonstrate no effect for lipid extraction other than the removal of the lipid epicuticle whereas detergents disrupt both the lipid epicuticle and at least some of the protein components of the underlying membranes.

The wings of mosquitoes remain intact after washing in distilled water at various temperatures for various periods. Illustrations are not given. The appearance of normal and control membranes is as shown in Figure 36. After soaking in distilled water, even at room temperatures, the membranes become less dense; this is shown particularly by an increase in contrast between the general membrane and the small darker (denser) areas.[11] Such increased contrast and decreased density is more marked with wing membranes soaked in hot water. It seems that some of the diffuse material is removed by water. Interpretation of the decreased density shown by electron micrographs of wing membranes is difficult. Resolution in these thick, multilayered membranes is relatively poor even in the best pictures, and accordingly any structural alteration would have to be relatively gross to be detected (at least several hundred Ångstrom units). Furthermore, wings consist of two membranes separated by the remnants of dead epithelial cells; it is conceivable that the material removed by water is largely this cellular debris. As far as can be determined from our pictures the wing membrane maintains its integrity. What

[11] The identity of these small dark areas has not been definitely established but it seems likely that they represent thickenings in the endocuticle comparable to those in cockroach tracheae.

PLATE IV



can be stated definitely is that these wing membranes do not become altered in water in a manner comparable to or to the extent that cockroach and bee tracheae do.

The soft, non-sclerotized abdominal cuticle of mosquito larvae was tested as an example representing approximately the other extreme from wing membranes. Normal structure is as shown in Figure 39. Soaking isolated, cleaned cuticles for one day or one week in distilled water at 20° C. produced no positively indentifiable alterations but again it should be stressed that resolution in such thick membranes is not very good (probable order of several hundred Ångstrom units). Soaking isolated, cleaned cuticles for one day or one week in distilled water at 65° C. produced marked changes; the results were not entirely constant but were all in the intermediate range between patterns shown by Figures 40 and 42. Treating living larvae with water at 65° C. (which is lethal) for a minute and then removing the cuticle and brushing it clean gave preparations indistinguishable from the controls, but treating such intact larvae with water at 65° C. for an hour produced a fibrous appearance comparable to that obtained by soaking isolated cuticles in water at this temperature. Soaking isolated cuticles for one day or one week in distilled water at 95° C. gave pictures more nearly like Figure 44. No interpretation is made for these results beyond saying that there are obvious structural changes following treating or soaking at 65° and 95° C. but that there are no clearly demonstrable changes following soaking at 20° C.

The above results may be compared with the data published by Fraenkel and Rudall (1940, 1947). Soaking in water at room temperatures causes very little

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PLATE IV

All figures reproduced at 16,000 ×

FIGURE 33. Cockroach, crop lining. Normal, washed in water. Dark structures are heavy projections. (No. 81b)

FIGURE 34. Cockroach, crop lining. Treated with conc. KOH at 160° C. for 15 minutes washed in water. (No. 81c)

FIGURE 35. Cockroach, crop lining. Treated with diaphanol at 25° C. for 41 weeks. (No. 251c)

FIGURE 36. Mosquito (*Aedes aegypti*), wing. Treated with 5 per cent KOH at 100° C. for one day. Not significantly different in appearance from control. The scattered darker areas appear consistently on normal and water-washed mosquito wing membranes but their identity has not yet been established. (No. 90d)

FIGURE 37. Mosquito, wing. Treated with conc. KOH at 160° C. for 15 minutes, washed in water. (No. 91b)

FIGURE 38. Mosquito, wing. Treated with 20 per cent KOH at 100° C. for one day, washed in water. (No. 91a)

FIGURE 39. Mosquito larva (*Aedes aegypti*), abdominal cuticle. Normal, washed in water. (No. 372e)

FIGURE 40. Mosquito larva, abdominal cuticle. Treated with 20 per cent KOH at 65° C. for 48 hours, washed in 95 per cent ethyl alcohol, then in water. (No. 368e)

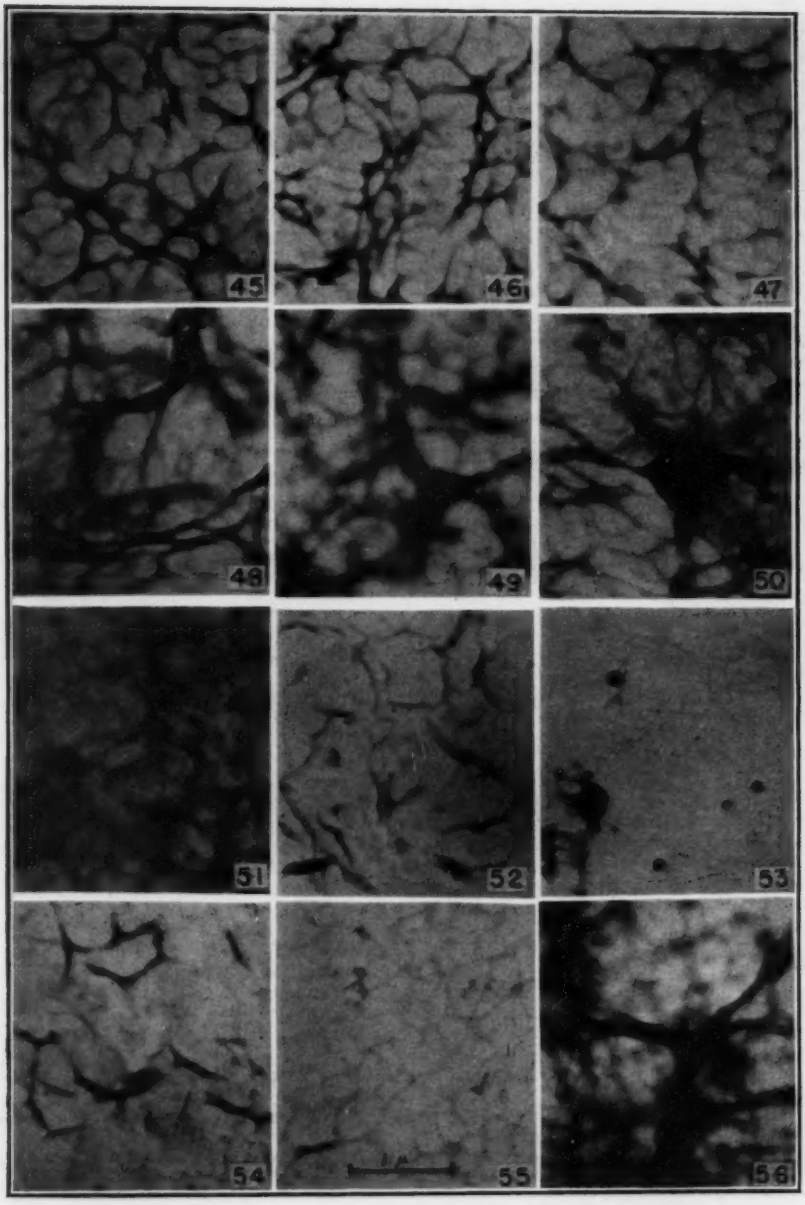
FIGURE 41. Mosquito larva, abdominal cuticle. Same treatment but washed only in 95 per cent ethyl alcohol. (No. 368d)

FIGURE 42. Mosquito larva, abdominal cuticle. Same treatment but washed only in water. (No. 367b)

FIGURE 43. Mosquito larva, abdominal cuticle. Same treatment but washed in water, then in 95 per cent ethyl alcohol. (No. 367d)

FIGURE 44. Mosquito larva, abdominal cuticle. Same treatment but washed in water, then in 95 per cent ethyl alcohol, then in water. (No. 368b)

PLATE V



loss of weight and no detectable change in electron micrographs. Soaking in water at high temperatures causes considerable loss in weight (at least largely removal of protein) and more or less extensive alteration of structure shown in electron micrographs.

All of the above examples are free from gross discontinuities (e.g., pore canals, gland ducts). The electron micrographs show that under favorable conditions changes that would invalidate permeability data do not occur at least down to the range of a few hundred Ångstrom units. However, most molecules of interest in studies of cuticle permeability are much smaller than this. Electron microscope studies show that numerous procedures introduce alterations that invalidate the use of such treatments of isolated cuticle but resolution in electron micrographs of cuticle is not fine enough to permit saying that no significant alterations occur after those treatments for which we could detect no changes. Other tests should also be used (e.g., change in membrane potential on soaking, etc.) and even with all possible checks final validation of the precise measurements possible with isolated cuticle will have to depend both on absence of demonstrable change and integration of the results with data from intact animals.

Discussion

The purification of chitin in membranes usually leads to extensive structural alterations demonstrable by electron microscopy. It follows that such purified chitin membranes can have no real biological significance in permeability studies. It also follows that we cannot continue to hold the old view that arthropod cuticle consists of a framework of chitin in which other components are merely embedded (see Fraenkel and Rudall, 1940, 1947; Richards, 1947 a, b).

Of more interest is the unexpected degree of dissimilarity of chitin patterns given by different types of cuticular membranes. The pattern obtained in a specific case is primarily correlated to the type of cuticle used rather than to the chemical treatment, and results of chitin purifications are reproducible with reasonable quali-

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Plate V

Honey bee (*Apis mellifica*), abdominal air sac. All figures reproduced at 16,000 ×

Figure 45. Normal, washed quickly in distilled water. (No. 229a)
Figure 46. Soaked in distilled water at 25° C. for 2 days. (No. 238e)
Figure 47. Soaked in isotonic saline at 25° C. for 2 days. (No. 253c)
Figure 48. Refluxed with boiling chloroform for 2 hours. (No. 228e)
Figure 49. Soaked in distilled water at 65° C. for 2 days. (No. 222a)
Figure 50. Soaked in distilled water at 65° C. for one week. Note differences between right and left halves indicative of more extraction from some areas than others. (No. 222e)
Figure 51. Soaked in distilled water at 100° C. for one day. (No. 323e)
Figure 52. Soaked in distilled water at 100° C. for 8 days. (No. 345b)
Figure 53. Soaked in distilled water at 100° C. for one month. Membrane so delicate that detectable in electron micrographs principally by small particle of debris on it. (No. 370d)
Figure 54. Treated with 10 per cent pepsin in acid solution at 36° C. for one day, washed in water. (No. 225c)
Figure 55. Refluxed with boiling chloroform for 2 hours, washed through acetone to water, and then treated with 10 per cent pepsin in acid solution at 36° C. for one day. (No. 228b)
Figure 56. Soaked in 0.02 per cent "Aerosol OT" (di octyl sodium sulfo succinate) in distilled water at 25° C. for 24 hours, washed in water. (No. 288a)

tative precision. Even though one can alter the pattern considerably after the purification treatment (alcohol versus water wash), we have not been able to obtain a single type of pattern from all types of cuticle by variations in technique. Depending on the type of membrane used, one obtains either a fibrous mat of greater or lesser degree of orientation, a vesicular membrane which is partly a fibrous feltwork, a reticulate membrane without distinct fibers or a seemingly homogeneous membrane with some small holes. If the continuous sheet in cockroach tracheae is found to be chitin we would have to add to this list a membrane homogeneous to less than 50 Å, at least when dry. These differences are not functions of membrane thickness and not functions of per cent de-acetylation of chitin to chitosan. Suggestions for an explanation of these diverse results would include: (a) a function of the relative percentage of chitin and protein in the membrane; (b) a function of degree of sclerotization; (c) a function of alkali-insoluble impurities; and (d) a function of different degrees of chitin polymerization. All four suggestions imply that chitin molecules vary either in size or in intermolecular bindings, and therefore they overlap and become aspects of one another.

The possibility of these diverse patterns being caused simply by variations in percentage of chitin present seems to us unlikely. There is really no convincing evidence for this statement. One can readily imagine variation in chitin percentage affecting the number of fibers per unit area, but we do not see how chitin percentage *per se* could account for fibrous networks versus homogeneous membranes, especially when the microfiber diameters (when found) are similar in different membranes, unless we also assume differences in cross linkages (which automatically moves the question to the fourth point).

Actually, the determination of the percentage of chitin present in any membrane is difficult, and we have only a crude idea of the accuracy of available methods (Campbell, 1929; Fraenkel and Rudall, 1947; Richards, 1947b). For most of the membranes studied in the present work it is not feasible to attempt such a determination. In current literature there are two opposed viewpoints on chitin-protein percentages in arthropod cuticle. Fraenkel and Rudall (1947) postulated a basically constant chitin percentage based on a hypothetical model of alternating monolayers of chitin and protein and assume that all deviations are secondary. Richards (1947) postulated a basically variable percentage of chitin based on the fact that all membranes have a high percentage of protein whereas the chitin percentage may range from 0 to 60 per cent. It seems to us difficult to reconcile our results with the alternating monolayer concept of Fraenkel and Rudall. The narrow range of microfiber diameters is readily interpretable on the idea of there being macromolecular chitin micelles which form mixed crystals with the protein molecules which have nearly identical lattice unit dimensions, but it would be surprising if monomolecular layers of chitin would reorient into macromolecular microfibers of such constant dimensions following removal of protein. Macrolayers in the cuticle are evident (e.g., interference color phenomena, alternating laminae in electron micrographs) but monolayers or even single micellar layers are not. At present all ideas are only working hypotheses.

The second possibility, variation in degree of sclerotization, is difficult to analyze. By definition, sclerotization is the formation of sclerites, i.e., hardened areas in the cuticle. Campbell (1929) was the first to show that this hardening is not due to chitin *per se*. Pryor (1940) elucidated part of the picture by demonstrating the

tanning of cuticular proteins by oxidized orthodihydroxyphenols with concurrent hardening. If this were the whole story of sclerotization the process should not affect the structure of purified chitin. But x-ray and other data lead to the assumption of chitin-protein bindings, the nature of which is not known. Our work shows that purification of chitin has less destructive effect on sclerotized membranes than on non-sclerotized membranes despite the fact that the sclerotized membranes contain lower percentages of chitin (Fraenkel and Rudall, 1947). It seems that as a result of sclerrotization the chitin molecule linkages are so changed that a different pattern is obtained following so-called purification of the chitin. Again we are led to the idea of variations in chitin cross-linkages to account for our diverse pictures even if the diversity is found to be a by-product of sclerotization.

The third suggestion, variable alkali-insoluble impurities, is at present only a conjecture. One of the great difficulties of work on cuticle is that we have no means of determining the degree of purification of chitin. It is quite possible that no one has ever seen pure chitin (note the low nitrogen value consistently obtained by Fraenkel and Rudall, 1947). Solution in acid leads to rapid hydrolysis of ether linkages (Clark and Smith, 1936) and it is not certain that solutions in lithium thiocyanate are free from impurities. Until we have conclusive analyses there is little that can be done about this uncertainty.

The fourth suggestion, various degrees of polymerization of chitin molecules, seems to fit our diverse pictures best but it cannot be claimed that our data prove this to be true. Chitin, unlike cellulose, is not known to occur naturally in anything approaching the pure state. It is customarily assumed that there must be chitin cross-linkages to or through other compounds but the possibility of various degrees of cross-linkages between chitin molecules seems not to have been suggested—no doubt largely because nothing definite is known about it. When microfibers are obtained they show a rather narrow range of diameters irrespective of whether they parallel one another or run randomly. This suggests there is some real significance to the values (micelle diameters ?), but the absence of such microfibers in certain purified membranes would seem to necessitate a different type or degree of cross-linkages between chitin chains or chitin micelles.

To account for our diverse electron microscope pictures there must be different degrees or kinds of reorientation of chitin molecules following removal of other membrane components. Unfortunately we can do no more than speculate about this at present. Conceivably such results might be a function of chitin chain lengths (if these vary like cellulose chains), or of stable cross-linkages present before purification, or of different spatial patterns in the cuticle tending to different degrees of chain cross-linkages (hydrogen bonds ?) following purification, or of variable amounts of stabilizing impurities if such exist. Apparently these linkages would not involve the acetyl group since purified chitin and chitosan membranes give similar electron micrographs. Obviously we need to know more about chitin chemistry on the polymer level.

Summary

1. Chitin purifications alter the structure of cuticular membranes of insects more or less extensively.

2. Our data agree with the x-ray diffraction studies of Fraenkel and Rudall in showing:

(a) The cuticle cannot be viewed as a rigid chitin framework in the interstices of which other components are deposited.

(b) Protein extraction methods have no value for localization of membrane components.

(c) Purified chitinous membranes have no significance for studies on the permeability of arthropod cuticle.

3. Several but not all types of membranes after purification yielded microfibers of chitin which after drying have diameters of $<$ 100 to 300 Å ($<$ 0.01 to 0.03 μ). It is suggested that these values may represent chitin micelle diameters.

4. Unexpected diversity in chitin patterns was obtained. Possible interpretations are discussed, and it is suggested that chitin cross-linkages vary considerably from one type of membrane to another type.

5. Chloroform and acetone remove the lipid epicuticle without affecting the structure of the underlying protein epicuticle and endocuticle. Detergents disrupt both the lipid epicuticle and underlying protein layers.

6. The effects of water and salt solutions on the structure of isolated cuticular membranes depend on temperature, the salt used and its concentration and the type of membrane. There is good correlation between weight losses recorded at various temperatures by Fraenkel and Rudall and structural changes found in our work. It is concluded that isolated cuticular membranes that are free from gross discontinuities can be used to a certain extent in permeability studies employing methods of physical chemistry but only when parallel tests show that the particular membranes remain reasonably near their original structure and composition.

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SEASONAL AND ANNUAL VARIATIONS IN THE ATTACHMENT AND SURVIVAL OF BARNACLE CYPRIDS[1]

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Two factors governing the development of a barnacle population have been investigated: namely, the number of barnacle cyprids attaching daily and the relationship of the daily cyprid accumulation to the number of adult barnacles attaching per month in Biscayne Bay, Miami Beach, Florida. On surfaces exposed for one month at this location, adult barnacles were invariably the dominant organism. No competition by other forms developed in this time interval to limit significantly the attachment of barnacles or their growth. This study was therefore concerned with numbers of cyprids settling daily, survival of cyprids, seasonal growth rates and space limitations, as factors affecting the size of the month-old barnacle population.

The survival of barnacles following attachment has been studied principally by Moore (1934), Hatton (1938) and Moore and Kitching (1939) with respect to the attachment of *Balanus balanoides* and *Chthamalus stellatus* to cleaned rock surfaces in the intertidal zone. These investigations were interested mainly in the effect of tidal level, exposure to surf and to sun, and interspecific competition on the growth and mortality of intertidal barnacles. Their findings are not directly applicable to barnacles attached to continuously submerged surfaces since they emphasize the effect of tidal exposure. The investigations of van Breemen (1933) on the survival of barnacles attached to continuously immersed surfaces considered the ultimate age of specific species.

Methods

To ascertain the numbers of barnacle cyprids attaching daily, a collecting surface of smooth, black glass was hung in the waters of Biscayne Bay at the Beach Boat Slips, Miami Beach, Florida, just below the low-tide mark. The cyprids which attached to one side of the glass (500 sq. cm.) during each 24-hour period were counted daily between 8:00 A.M. and 8:15 A.M. The surface was wiped clean after each counting. Temperature and density of the surface water were also determined at the same time. From these data salinity was calculated. The investigation was carried out over a period of 38 months, except for short interruptions in the summers of 1943, 1944 and 1945.

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During the same period, monthly fouling attachments in Biscayne Bay were also reported. These fouling collections were made on clear glass panels, backed with a dark red paint, of the same area as the cyprid collecting surface. The dark red backing provided a substratum color which was comparable to the black glass (Visscher and Luce, 1928). A total of 74 monthly collections were made, panels being immersed every 15 days for 30 days of exposure. From the monthly attachment of assorted fouling forms the barnacle count was extracted for comparison with the sum of the daily cyprid attachment for the same month. *Balanus improvisus* was the dominant adult barnacle on the monthly collectors and presumably was responsible for most of the cyprids (Weiss, 1948).

Results

The daily records of cyprid attachment, water temperature and salinity are presented in Figures 1, 2 and 3, respectively. Three-day moving averages were employed in plotting these data, for the purpose of minimizing random fluctua-

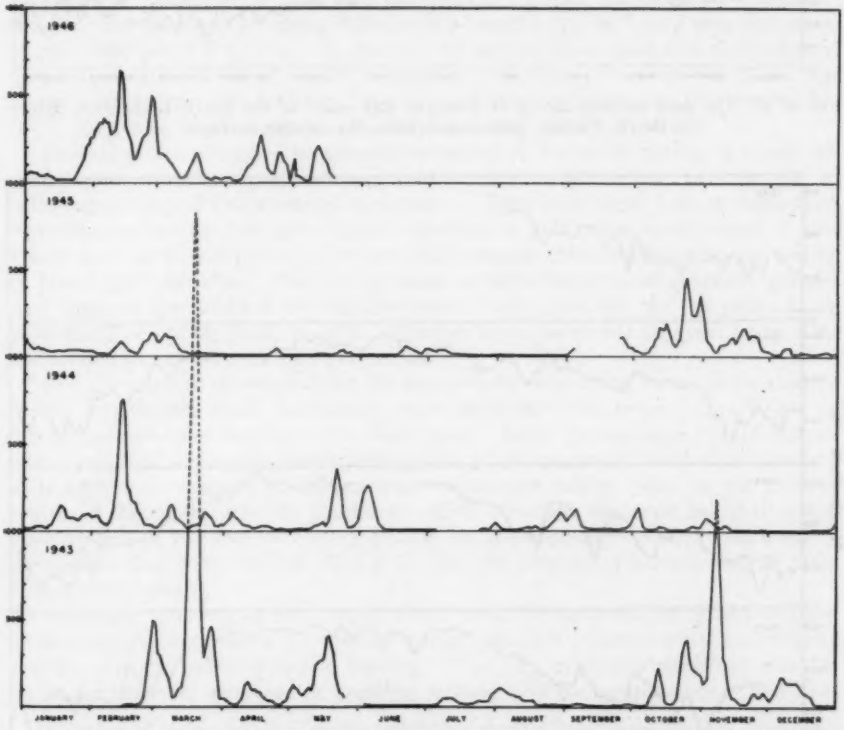

FIGURE 1. The daily accumulation of barnacle cyprids on black glass (500 sq. cm.) at the Beach Boat Slips, Miami Beach, Florida, February 1943–May 1946, plotted as a three-day moving average.

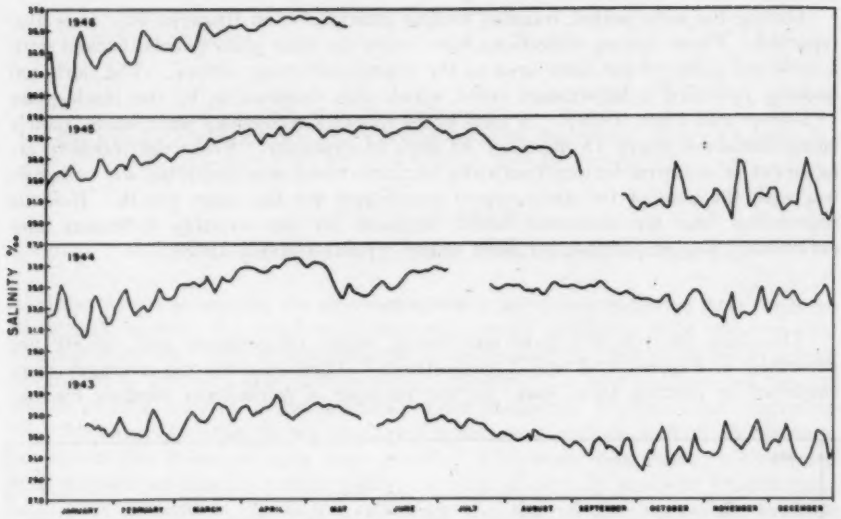

FIGURE 2. The daily salinity record of Biscayne Bay made at the Beach Boat Slips, Miami Beach, Florida, plotted as a three-day moving average.

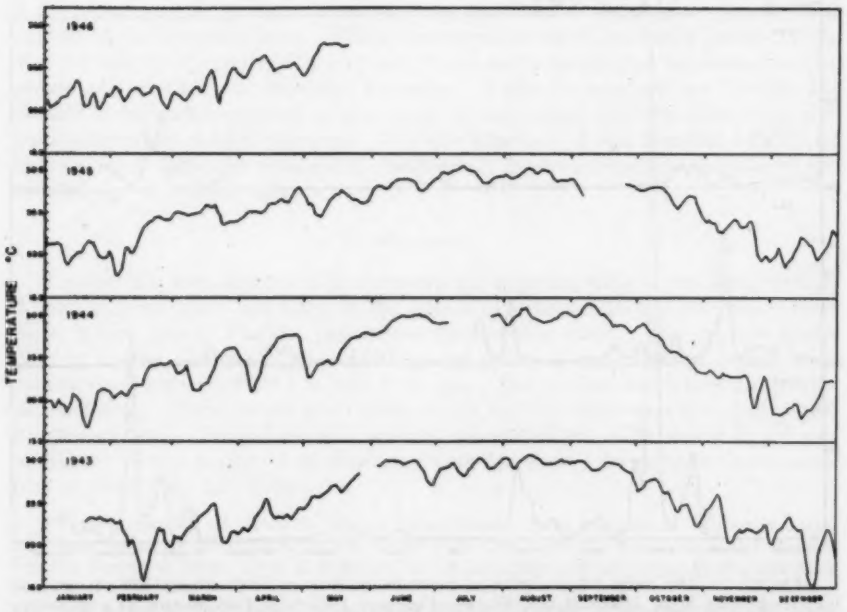

FIGURE 3. The daily temperature record of Biscayne Bay made at the Beach Boat Slips, Miami Beach, Florida, plotted as a three-day moving average.

tions. Both the temperature and salinity of Biscayne Bay showed seasonal variation. The temperature fluctuated each year between approximately 16° C. and 30° C. The salinity showed an annual range from approximately 28.00 $^{o}/_{oo}$ to 37.00 $^{o}/_{oo}$, the peak coinciding with the end of the dry season. This period occurred each year between April and June. With the onset of the summer rains, the bay salinity declined, reaching its minimum in the period from October to January. During this period, and to a lesser degree in the month preceding and following, the salinities showed marked short-period fluctuations, which were indicative of the stage of tide at which the water sample was taken. A sample taken at high tide was always more saline than one sampled at low tide. This fluctuation of salinity was most pronounced during the rainy season, when the surrounding land areas were well soaked and the fresh water run-off from the land diluted the bay water. Slight tidal variations in salinity were evident even in the period of maximum salinity, but the range was not as great. Temperature fluctuations paralleling the tidal cycle were also evident due to the more rapid warming and cooling of the comparatively isolated bay water.

Barnacle cyprids settled on every day but three during the 38 months of observation. The numbers attaching daily varied from as low as 1 or 2 to a maximum of over 2800 per 500 sq. cm. In general, the settling of cyprids was characterized by peaks in attachment at relatively frequent intervals. Nevertheless, there was no apparent relationship between the sizes of successive peaks and the interval between the peaks, which ranged in most cases from 7 days to a month.

Several peaks of cyprid attachment occurred in the early spring and late fall of each year, but these peaks showed considerable variation from year to year in both magnitude and exact time of occurrence. The period from June to September was characterized by low daily cyprid attachments and a few small peaks, if any. However, in 1945 this period of reduced daily attachment extended from the middle of March to September. The spring peaks of attachment were generally greater than those of the fall; but in 1945 this pattern was reversed, the fall peaks being greater. The spring peaks in 1946, although large, were not as great as in 1943 and tended to extend over a longer period.

For the purpose of ascertaining the mechanism controlling the periodic rises in cyprid attachment, these fluctuations were analyzed with respect to changes in the temperature and salinity of the bay water. Since the barnacle cyprid follows after a series of six nauplii stages, fluctuations of its attachment were also examined with respect to changes in the marine environment taking place at the time of release of the nauplii into the plankton. A search of the literature failed to reveal the duration of the free-swimming period for *Balanus improvisus*. However, of the species that were studied (Table I), the free-swimming periods ranged from 7 days to 3½ weeks.[3]

Assuming intervals of one, two or three weeks between spawning and settling, no satisfactory correlations were found between changes in temperature and salinity and the periodic peaks in cyprid settling. The only relationship evident was between the seasonal increases in numbers settling and the temperature. The rises

[3] The period of time that the cyprid spends in the plankton prior to settling was noted quantitatively by Willemoes-Suhm (1876), who studied the development of *Lepas fascicularis*. In addition to observing six nauplii stages, he noted that the "cyprid evidently attaches itself on the very first occasion."

TABLE I

Time for development through nauplii stages to cyprid

Barnacle species	Number of nauplii stages	Time for development to cyprid
Balanus crenatus Bruguiere [1]	6	7–10 days
Balanus crenatus Bruguiere [2]	8	13–14 days
Balanus eburneus [3]	6	7–10 days
Balanus perforatus Bruguiere [4]	6	21 days
Chthamalus stellatus [5]	6	18–20 days
Verruca stroemia [6]	6	23–27 days
Balanus balanoides [5]	6	3–3½ weeks

[1] Bohart (1929). [3] Grave (1933). [5] Bassindale (1936).
[2] Herz (1933). [4] Lochhead (1936).

in cyprid numbers in the spring and autumn occurred between the periods of minimum and maximum temperatures. This relationship suggested that *Balanus improvisus* breeds in a temperature range of approximately 18° C. to 27° C. The temperature above which breeding diminished greatly or ceased was more clearly defined than the minimum temperature limit. The waters of Biscayne Bay exceeded the upper temperature breeding limit for several months each year, but rarely and only for short periods did the temperature drop below the lower breeding limit.

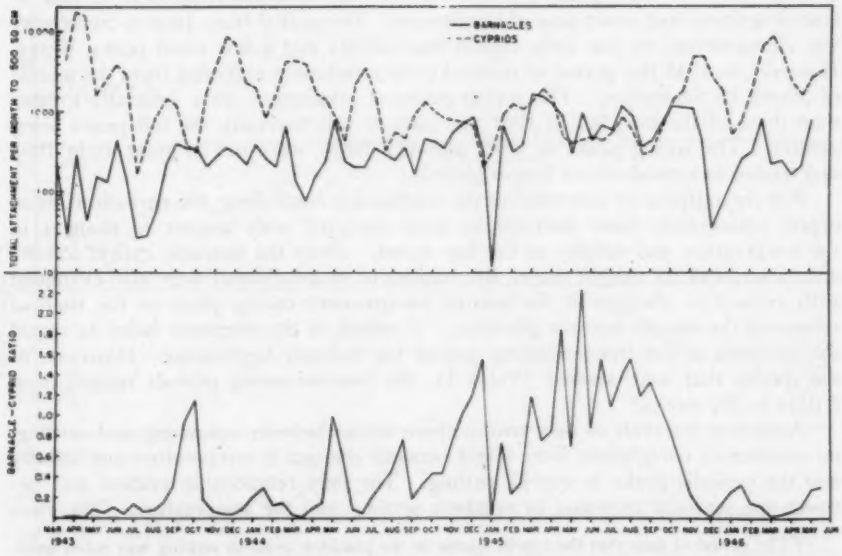

FIGURE 4. The monthly totals of daily cyprid settling and the monthly barnacle attachments on glass surfaces (500 sq. cm.), upper half. The ratio of barnacles to cyprids attached in each monthly period, lower half.

The numbers of barnacles found on the monthly collectors showed considerable variation, as indicated in Figure 4. For the entire period of observation the monthly barnacle collections dropped below 100 only eight times and went above 1000 only six times. The extreme numbers of barnacles collected in one month were 10 in December 1944 and 1615 in February 1945. However, extreme fluctuations in numbers of barnacles did not usually occur in two successive collections but followed a period in which a trend of rising or falling numbers was evident. Although not as marked, a rising trend in the monthly collections of barnacles during the period of 1943–1945 was observed, reaching a peak simultaneously with the low point of the monthly totals of cyprids.

Of the varying numbers of cyprids attaching daily it was assumed that all or some portion metamorphosed and grew to adult size. For comparison with the monthly barnacle collections the daily cyprid attachments were totaled every 15 days for the preceding 30-day period. The monthly cyprid totals, in general, showed a greater fluctuation than the monthly barnacle collections, as indicated in Figure 4. During the 38 months of observation, the numbers of cyprids attaching in a month decreased to a minimum in 1945 and then rose once more. This fluctuation is more clearly indicated in Table II, in which the number of monthly

TABLE II

Number of monthly cyprid totals greater and less than 1000 in six-month periods

Period	>1000	<1000
March 15, 1943–September 1, 1943	9	3
September 15, 1943–March 1, 1944	9	3
March 15, 1944–September 1, 1944	6	6
September 15, 1944–March 1, 1945	4	8
March 15, 1945–September 1, 1945	2	10
September 15, 1945–March 1, 1946	9	3

cyprid totals greater than 1000 are compared with the number of monthly totals less than 1000 for six-month periods. The number of monthly cyprid totals of less than 1000 reached a maximum in the period from March 15, 1945 to September 1, 1945. The concurrence of a high proportion of monthly cyprid totals less than 1000 with a period of maximum barnacle collections suggests that conditions for survival and maturity of the cyprid were optimum when the daily rate of attachment totaled 400 to 1000 per month (Fig. 4, upper half).

However, there were five occasions when the monthly cyprid totals in this range were not paralleled by monthly barnacle collections indicative of good survival. Since three of these collections were in the summer and two in the winter, it was not possible to identify these anomalies with a particular temperature condition or any other specific phenomenon. Cyprid totals of 2000 or more, which were found in 25 out of 74 monthly summations, invariably resulted in high mortality and poor survival. These data have been graphically presented by Deevey (1947) in a recent review of natural populations of animals. The phenomenon of attachment of enormous numbers of cyprids and high mortality was attributed by Moore (1935) to depletion of the stock of food in the water by the extra-large pop-

ulation. It should be pointed out that there were several occasions, particularly in 1945, when monthly cyprid totals slightly greater than 1000 were paralleled by large barnacle collections.

The ratio of the monthly totals of barnacles to cyprids is presented in the lower half of Figure 4. As shown, a ratio of 1 indicates survival of all attaching cyprids, and values less than 1 indicate the degree of survival (and mortality) of the cyprid population. In addition, there is the anomalous condition of ratios greater than 1, which indicates more adult barnacles at the end of a month than the total of settling cyprids in the same period.

The survival ratio was greater than 1 in October 1943, May and December 1944, and on nine of the monthly collections in 1945. A satisfactory explanation of this unusual condition may be found in the thesis of Phelps (1942) and of Scheer (1945) that the bacterial slime film, which forms on surfaces submerged in the sea, provides an environmental condition more suitable for attachment and growth than a clear bare surface. The daily collector, wiped clean every morning, may not have been as attractive a surface for attachment as the collector continuously immersed, which permitted a slime film to form within a few days after immersion.

During the period of observation, a distinct tendency toward greater survival of the cyprids was observed, reaching a peak in 1945 and then decreasing sharply. Although occasional rises of the barnacle-cyprid ratio were indicative of good survival in 1943 and 1944, the year 1945 was by far the best survival year.

Another factor limiting the survival of barnacles was the area available for growth on the collecting surface. The maximum adult population, one month old, was obtained during February 1945 when 1615 barnacles filled the collector. On many other occasions, particularly during the summer months when the growth rate was highest (Weiss, 1948), considerably fewer barnacles, for example 525 in June 1945, filled the same collecting area. Thus the area available for barnacle attachment was limited by the rate of growth of the barnacles initially attached.

Summary

1. The daily barnacle cyprid attachment in Biscayne Bay, Florida, and concomitant water temperature and salinity measurements were recorded continuously during a period of 38 months.

2. No relationship between the frequent peaks in cyprid settling and changes in temperature and salinity was established.

3. Seasonal periods of heavy cyprid attachment were observed to occur within a temperature range from 18° C. to 27° C.

4. The survival of cyprids increased gradually from 1943 to 1945 and then decreased sharply, as shown by a comparison of monthly barnacle collections with monthly totals of daily cyprid attachments.

5. Seasonal variation in growth rates was observed to be an important factor in limiting the numbers of barnacles attached per unit area.

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A STUDY OF THE REPRODUCTIVE ORGANS OF THE COMMON MARINE SHRIMP, PENAEUS SETIFERUS (LINNAEUS)

JOSEPH E. KING[1]

Introduction

In connection with the investigations of the common commercial shrimp, *Penaeus setiferus* (Linnaeus), carried on by the U. S. Fish and Wildlife Service, studies have been made of the structure of the reproductive organs and accessory sex characters and the general nature of the reproductive process.

This report gives information on the anatomy and histology of the male and female sex organs, the maturation of the germ cells, and certain observations on the phenomena of impregnation and ovulation.

This study was undertaken to determine if there are structural characters within the gonads which might serve as an index to age and longevity and furnish information bearing on the frequency of spawning of an individual within a single breeding season. Such data are needed for estimating mortality rates and for an understanding of the reproductive potential of the organism.

Materials and Methods

The observations which follow are based upon the macroscopical and microscopical examination of the gonads and accessory organs of several hundred shrimp taken at irregular intervals over a period of several years. Because of various circumstances, it was necessary to obtain much of the material from the commercial catch. These shrimp had been iced on the fishing grounds immediately upon capture and after 8 to 10 hours were still in a satisfactory state of preservation for general histological study. Much of this material, of course, was not suitable for detailed cytological work. When opportunity permitted trips were made to the fishing grounds and fresh material obtained.

At the beginning of the study the entire gonads of several specimens of each sex were serially sectioned and examined to determine if the various parts of the organ were homogeneous in structure and stage of development of the germ cells. As in general they were found to be so, samples, thereafter, were taken of only a portion of the gonads. In the female, this was usually the abdominal extension of the ovary lying in segments XVII and XVIII. In the male two or three lateral lobes of the testis were preserved from each specimen, together with one entire vas deferens and terminal ampoule.

Two generally different microscopical techniques were employed: in the first; tissues were prepared for general histological study; in the second, ovarian material was treated so as to preserve its true chemical nature for cytological study. In the first method, the tissues were fixed in either Bouin's or Kahle's fluid, imbedded in

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paraffin and stained with Delafield's Hematoxylin (Harris modification) and Eosin (1 per cent in dioxane) or Mallory's triple connective tissue stain. In this method dioxane was used in the imbedding and staining operations.

In the second method, the tissues were fixed in 20 per cent formalin, sectioned with a freezing microtome and stained with one of the following fat-specific stains: Sudan IV, Calco Oil Red, Calco Oil Blue or Oil Yellow OB. Sections obtained by this method were also treated with ninhydrin, a protein-specific microchemical reagent.

The sexes of *P. setiferus* are easily distinguishable. In the male the endopodites of the first pair of pleopods are modified to form a copulatory organ, the *petasma* (Plate C, 2 and 3). The corresponding abdominal appendages of the female do not show this modification but are similar to the other pairs of pleopods. Additional but less obvious differences between the sexes include the locations of the openings of the genital ducts, the possession by the female of a ventral thoracic structure, the *thelycum* (Plate A, 3), the slight modification of the second pair of pleopods in the male (Plate C, 4), and significant differences in total length attained. As an example of the latter, total length measurements made on April 15, 1942, of a random sample of 200 adult shrimp of the offshore commercial catch gave an arithmetic mean of 165 mm. for 110 males and 171 mm. for 90 females. The males ranged in length from 147 to 178 mm. and the females from 151 to 188 mm., with the corresponding modes at 165 mm. and 176 mm.

In the Penaeid shrimps, as contrasted with the Pandalidae (Berkeley, 1929), no cases of sex reversal have been noted. The spawned eggs are released free in the water and are not carried on the pleopods of the female as in most other Decapods.

The Female Reproductive System

Gross Anatomy

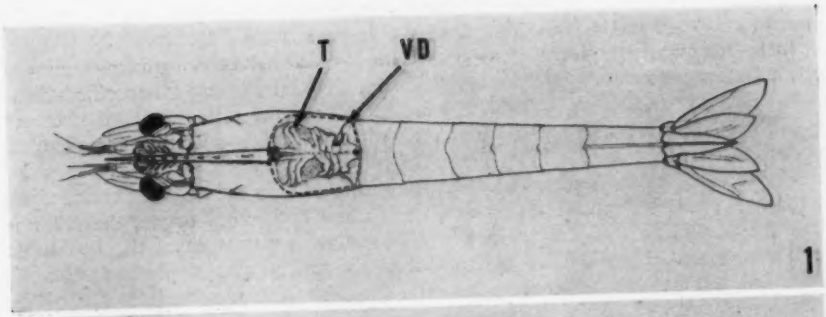
The female reproductive system (Plate B, 2 and Plate A, 2) consists of paired ovaries and oviducts and a single thelycum. The ovaries are partly fused, bilaterally symetrical bodies extending in the mature animal for almost its entire length, actually from the cardiac region of the stomach to the telson. In the cephalothoracic region each organ bears a slender anterior lobe and in most cases seven finger-like lateral projections. A pair of lobes, one from each ovary, extend the length of the abdomen.

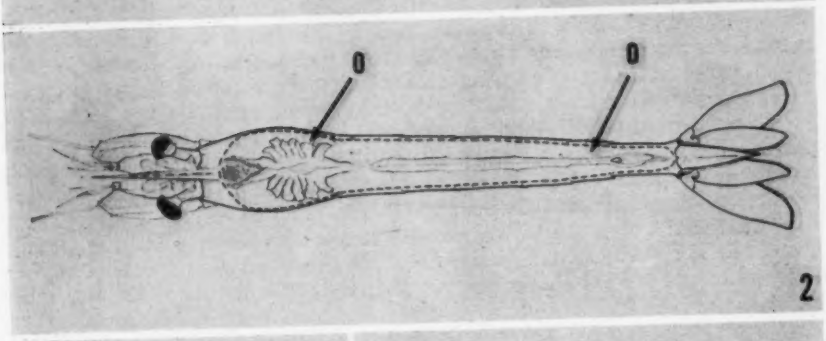
The anterior lobes lie closely applied to the esophagus and cardiac region of the stomach. The lateral lobes are located dorsally to the large mass of hepatopancreas and ventrally to the pericardial chamber. The heart rides like a saddle over this portion of the gonad. The abdominal extensions lie dorso-lateral to the intestine and ventro-lateral to the dorsal abdominal artery.

Fortunately for the observer in the field, maturation is accompanied by distinctive color changes in the ovary as well as changes in its size, which make it possible for one to identify the stage of development with fair accuracy without microscopic examination. The ovaries of the young shrimp (Plate D, 5) are so small in size and transparent in nature that their dissection in the fresh specimen is particularly difficult. Such a shrimp is recorded as *U* for undeveloped.[2] As the shrimp grows,

[2] Early in the history of the Investigations it was decided that the symbols *U, D, V, R* and *Sp* were to be used by field workers in designating the obvious stages of ovarian development.

PLATE A





the glands increase in size, scattered melanophores appear over the surface, and in general appearance, the ovaries become somewhat opaque. This stage is classed as *D*, or developing (Plate D, 3 and 4). As maturation continues, the ovary takes on a yellowish cast which deepens to a yellowish-orange, and is designated as the *Y*, or yellow stage (Plate D, 2). This is followed by the drab olive-brown color of the ripe, or *R* ovary (Plate D, 1). The ovaries, at this point, are so distended that they now fill all available space and appear to crowd considerably the other organs of the body cavity. During the yellow and olive-brown color phases, the ovary can be seen quite clearly through the abdominal tergites and membraneous connectives between the first abdominal somite and the carapace. The recently spawned ovary, designated as spent (*Sp.*), has collapsed from its distended condition and is not as deeply colored as in the ripe phase. As regression continues, the green color disappears but the structure remains opaque. A shrimp in this stage is difficult to distinguish from the large shrimp of the O-year class with developing ovaries. Microscopical examination, however, reveals the difference in most cases.

The oviducts (Plate B, 2) are short, narrow tubes leaving the ovaries at the tips of the 6th pair of lateral lobes and opening through the genital pores at the bases (coxae) of the 3d pair of pereiopods (Plate A, 3). Each opening is concealed in a bristle-filled, ear-shaped protuberance.

From a survey of the literature (Andrews, 1911; Burkenroad, 1934; Heldt, 1938; Filbo, 1943) it appears there are several ideas as to exactly what constitutes the thelycum (Plate A, 3). In *P. setiferus* it might be described as modifications of the sternal surfaces of somites XII, XIII, and XIV, which provide for the attachment of the spermatophore received from the male during copulation. In this species there are principally three things responsible for the retention of the spermatophore:

1. When discharged by the male, the spermatophore is accompanied by a mass of glutinous material (Plate B, 4 and 5) which is an important factor in its adherance to the female.

2. On the coxae of pereiopods four and five of the female there are protuberances bearing clumps of stiff bristles (Plate A, 3 and 4) which are directed medially and overlap flanges of the spermatophore, thus helping to hold it in place.

3. The "wings" of the spermatophore are securely anchored in a groove in the sternal surface between the 3d and 4th pereiopods (Plate A, 4). The horn-like structure on the posterior margin of each wing appears to be inserted between the bristle-bearing protuberance of the coxae of the 4th pereiopod and a raised shelf-like portion of sternite XIII.

PLATE A

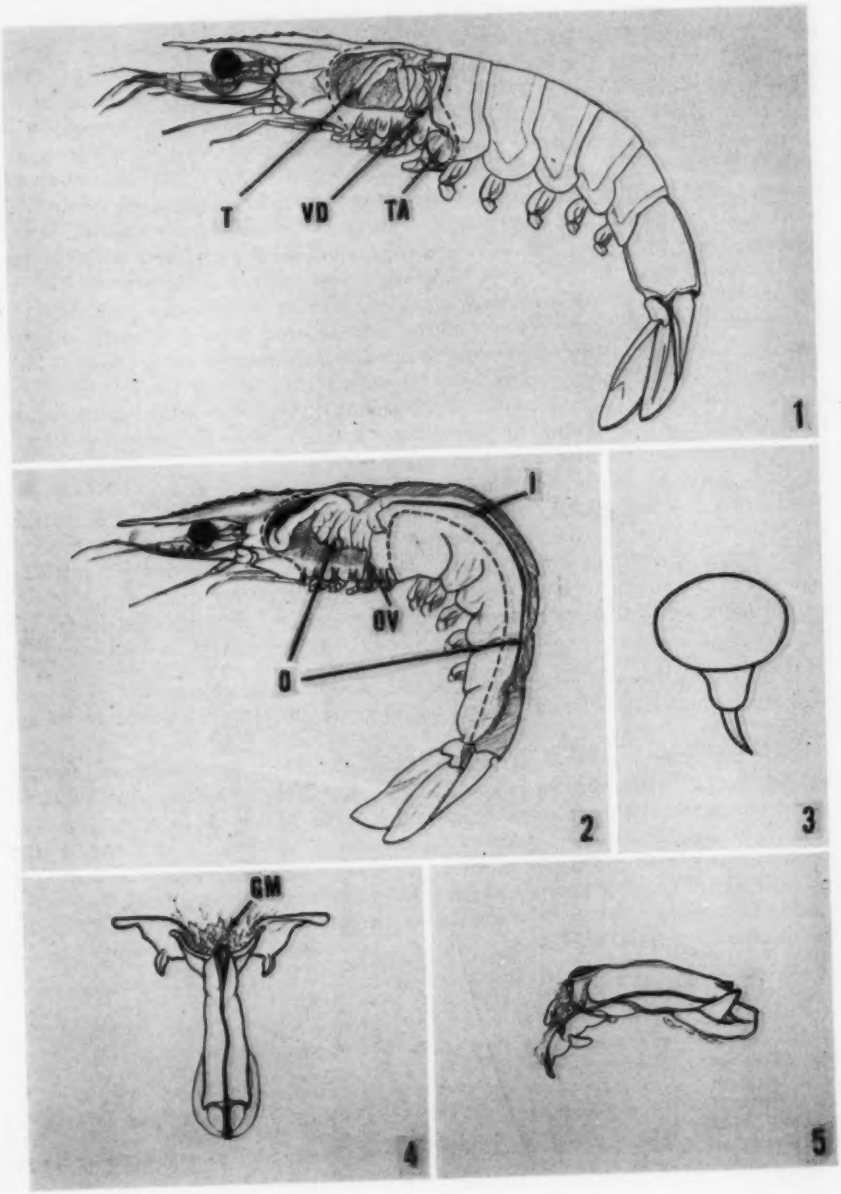
1. Diagram of male, dorsal view, dissected to show testes and portions of the vasa deferentia. T—testis; VD—vas deferens. × 0.5.

2. Diagram of female, dorsal view, dissected to show ovaries. O—ovary. × 0.5.

3. Diagram of ventral surface of cephalotharax of female. OP—opening of oviduct; TH—thelycum. × 2.5.

4. Diagram of ventral surface of cephalotharax of female with spermatophore attached. S—spermatophore. × 2.5.

PLATE B



On somite XIV are a pair of light-colored, pad-like structures which in *P. setiferus* play no active role in the impregnation process. In most other Decapods, however, these structures are open at their anterior margins, thus forming pockets that function as seminal receptacles.

Despite these holdfast devices, the spermatophore is rather easily dislodged, which may, to a certain extent, explain why spermatophore-bearing females are not commonly taken (Weymouth, Lindner and Anderson, 1933).

Histology and Development

The ovary

The wall of the ovary is composed of three layers: a thin outer layer of pavement epithelium, a relatively thick layer of connective tissue, and an inner layer of germinal epithelium. No layers of muscle fibers could be detected with the Mallory's triple staining technique. Extending longitudinally throughout the ovary are septa, also of connective tissue. Small blood sinuses can be seen in most cross-sections.

Germinal epithelium, functioning as such and giving rise to oogonia, is not distributed uniformly over the inner wall of the ovary but is confined to a certain well-defined area which has been termed the "zone of proliferation" (Gutsell, 1936). In the abdominal extension of the ovary there is a streak of such germinal epithelium along the medial-ventral wall of each lobe (Plate D, 1, 2, and 3; Plate E, 2, 3, and 4). In the lateral lobes this tissue occurs in a more strictly ventral position.

As the oogonia pass through the developmental stages of primary oocyte and secondary oocyte, they move in a column from the "zone of proliferation" toward the center of the ovarian lobe. Because of the rapid multiplication of cells, from this point the oocytes are forced to the peripheral regions of the lobe. As development proceeds, the peripheral and more mature oocytes are surrounded with "nurse" or follicle cells (Plate E, 3) which appear to arise from the germinal epithelium. Follicle formation continues until, in the ripe ovary, all the mature ova are enclosed by these nutritive cells.

The cytoplasm of the young ova, of *U* and *D* ovaries, appears finely granular with the staining methods used. As the egg matures these fine particles become enlarged and globule-like. In the *R* ovum we find very prominent rod-like bodies (Plate D, 6) imbedded in the cytoplasm in the peripheral regions of the cell and arranged radially about the nucleus.

With the Hematoxylin-Eosin staining technique, the cytoplasm of the *U* and *D* ova takes on a blue color indicating a basophilic reaction. As the egg matures into

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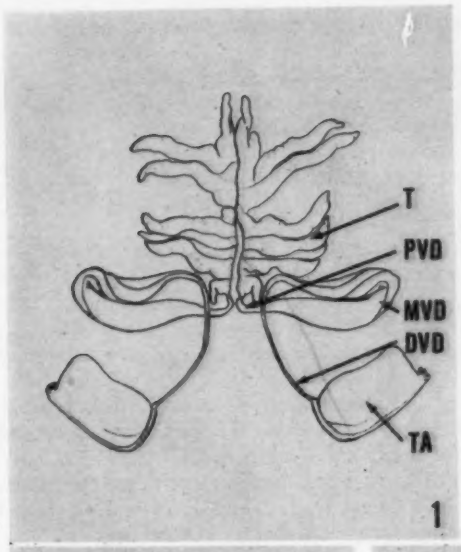
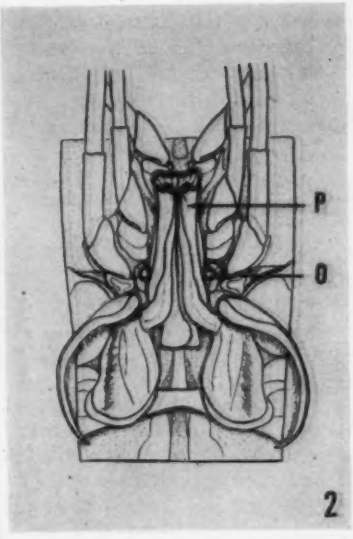
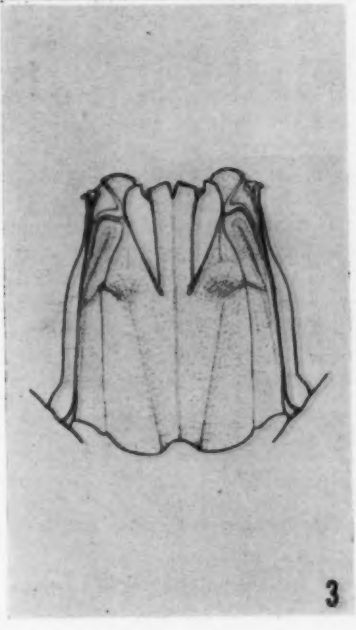
PLATE B

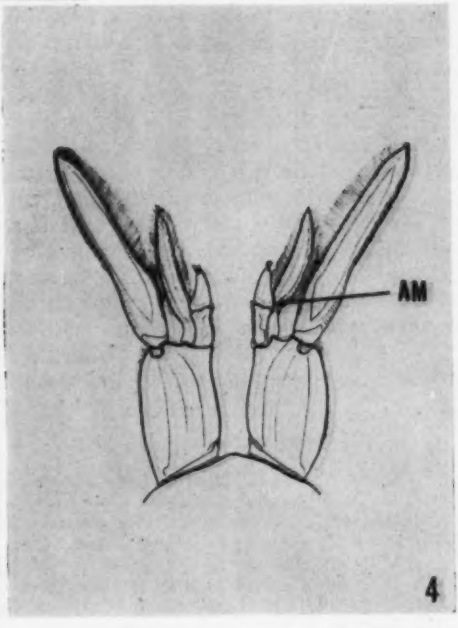
1. Diagram of male, lateral view, dissected to show reproductive organs. T—testis; VD—vas deferens; TA—terminal ampoule. × 0.5.
2. Diagram of female, lateral view, dissected to show relationship of ovary and oviduct. O—ovary; OV—oviduct; I—intestine. × 0.5.
3. Diagram of shrimp spermatozoan. × 9000 (approx.).
4. Diagram of ventral view of spermatophore (as in attached position). GM—gelatinous material. × 2.75.
5. Diagram of lateral view of spermatophore. × 2.75.

PLATE C









the *Y* and *R* stages, the cytoplasm becomes acidophilic, staining red with Eosin. The rodlike peripheral bodies just described also stain red with Eosin.

Through the use of fat-specific dyes, such as Sudan IV, Calco Oil Red, Calco Oil Blue and Oil Yellow OB, it was found that no fat was present in the *U* ovaries, a very little in the more advanced *D* ovaries, and a great amount in the *Y* and *R* stages. The presence of fatty yolk in significant quantities coincides, therefore, with the appearance of the yellow color in the ovary.

In the ripe ovum the yolk globules reacted strongly with all of the fat soluble dyes used. The peripheral bodies, however, did not take on the dye, but appeared as distinct clear areas in the cytoplasm. It was concluded, therefore, that they are not of fatty nature.

Sections of ripe ova were treated also with ninhydrin, a protein-specific reagent, to determine if the rod-like bodies might be albuminous yolk. No reaction was obtained. Bhatia and Nath (1931) have observed very similar cell characters in the ova of *Palaemon lamarrei*, another species of shrimp. They believed the bodies to be albuminous yolk derived from mitochondria.

The recently spawned ovary is easily distinguishable from the ripe stage. It is flaccid rather than turgid, of a muddy green color, and microscopical examination reveals numerous ripe eggs undergoing resorption. As the degenerative process continues the gland becomes greatly reduced in size and assumes an opaque milky color. At this time it is very difficult to distinguish from an ovary in a late *D* stage. By taking into consideration such factors as total length, weight, and time of year, it is usually possible to segregate these groups in the field. When examined microscopically it is found that the two ovaries have a distinct differentiating character. The spent ovary is fairly swarming with left-over follicle cells (Plate D, 8). In the late *D* stage (Plate E, 3) follicle formation is just beginning and these cells are present only in small quantities.

A careful study of the spent ovary has been made to determine if it might be used as an index to the shrimp's age and number of times spawned. Although space in the ripe ovary is almost entirely given over to the crop of mature eggs, the "zone of proliferation" is still evident and a few young oocytes are continually being formed. Spent ovaries, three to four months after spawning, have undergone considerable regrowth. Even as early as March spent ovaries have been examined which showed evidence of regrowth. On this basis it appears quite possible for a shrimp spawning in March to spawn again later in the season. In view of the long spawning season, which in Louisiana offshore waters is March through September (Anderson, Lindner and King, 1948a), sufficient time may remain for the second crop of eggs to mature. We have been unsuccessful, however, in finding

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PLATE C

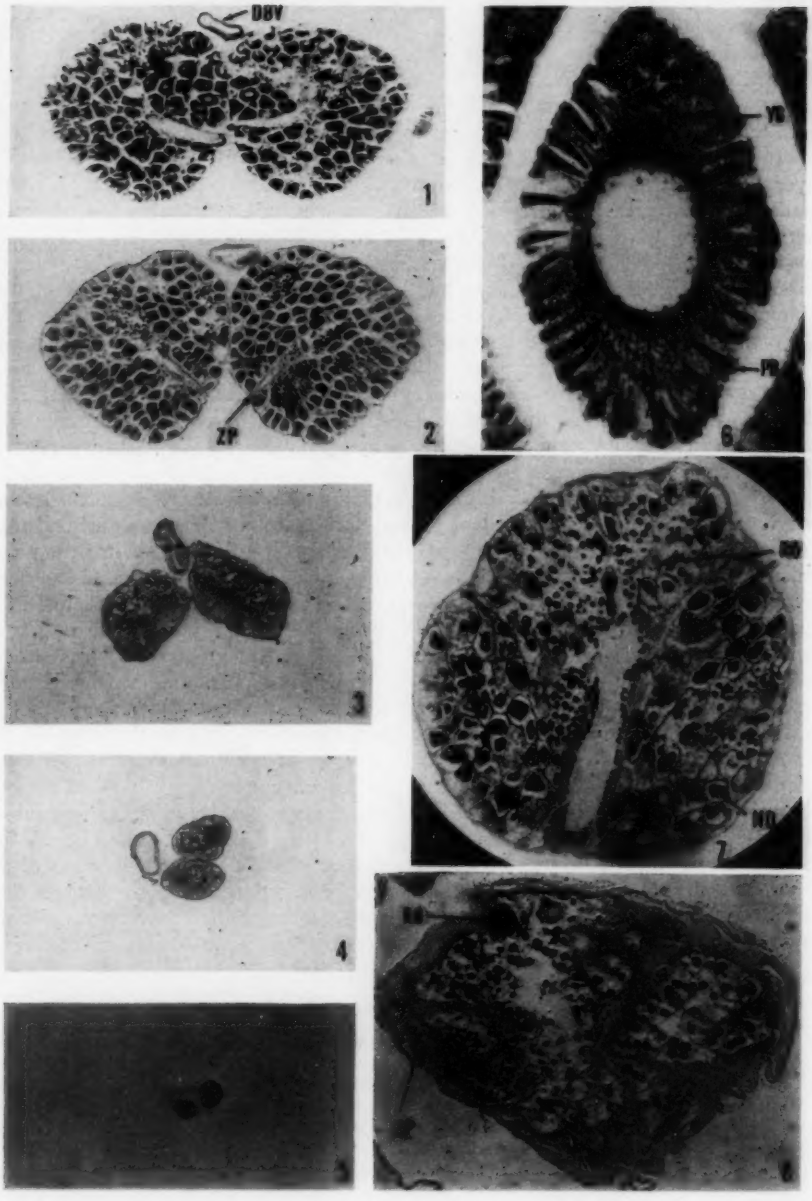
1. Diagram of male reproductive system. T—testis; PVD—proximal vas deferens; MVD—medial vas deferens; DVD—distal vas deferens; TA—terminal ampoule. × 1.75.

2. Diagram of ventral surface of mature male. P—petasma; O—opening of vas deferens. × 1.75.

3. Diagram of petasma of mature male spread open to show interior arrangement of folds. × 2.9.

4. Diagram of second pair of pleopods of male. AM—appendix musculina. × 1.75.

PLATE D



any ovarian characters such as are present in the higher animals which would enable us to distinguish between the once-spawned and the twice-spawned ovary.

It was hoped that the structure of the ovary would supply clues as to the approximate age and length-of-life of the shrimp. This possibility was removed by the likelihood of multiple spawning and the absence of any permanent "scars" or walled-off spent areas within the ovary resulting from each spawning. For our knowledge of the longevity of the shrimp, therefore, we have had to rely upon information obtained through length frequency studies (Anderson, Lindner and King, 1948b, 1948c). These data have shown that young-of-the-year shrimp are dominant in the commercial catch after August of each year. By October and November, old of the previous year are very scarce and form a small percentage of the commercial catch. It is possible to follow through a trace of this old group until about the middle of January, after which they either die out or become merged with the following year class so that, with our present information, they cannot be distinguished (Lindner, Anderson and King, 1948). By the middle of January these old shrimp are approximately 1½ years of age. If shrimp live beyond this point, they are at least not found in the commercial catch much beyond December and not at all beyond the middle of January.

The oviduct

The wall of the oviduct (Plate E, 6) appears to be made up of three layers. Bordering on the lumen is a somewhat folded columnar epithelium which, it is assumed, secretes a lubricating fluid to facilitate the passage of eggs down the tube. The main supporting structure of the duct is a thick layer of connective tissue. Although not apparent in cross-section, the outer layer would consist of a thin epithelial membrane. No muscle tissue was shown to be present by the Mallory's triple staining technique.

Ovulation

It does not appear that nature has made the proper provision for the egress of the half million or so eggs which the average female will produce. As mentioned

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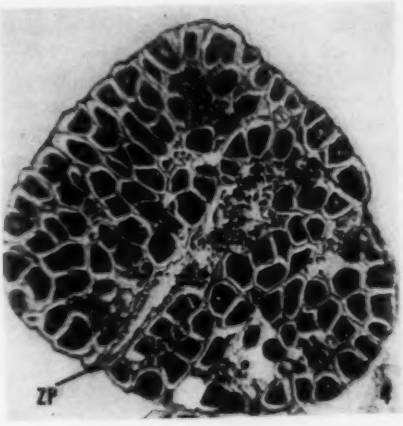
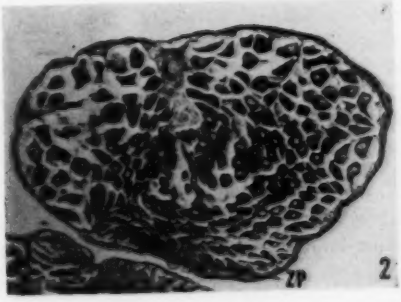
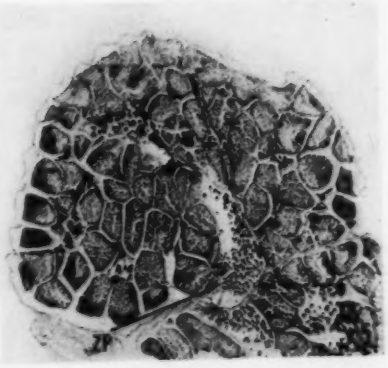
PLATE D

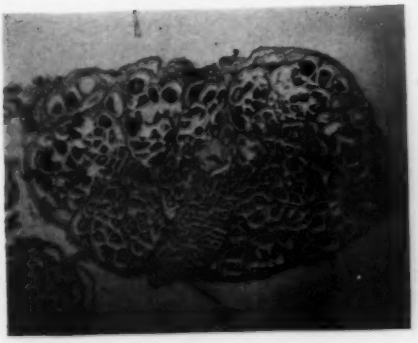
1. Ripe (R) ovary from 195 mm. shrimp taken March 7, 1944, cross-section of abdominal lobes. DBV—dorsal blood vessel. × 14.
2. Yellow (Y) ovary from 200 mm. shrimp taken March 7, 1944, cross-section of abdominal lobes. ZP—zone of proliferation. × 14.
3. Developing (D) ovary from 191 mm. shrimp taken December 15, 1944, cross-section of abdominal lobes. × 14.
4. Early developing (D) ovary from 180 mm. shrimp taken December 15, 1944, cross-section of abdominal lobes. × 14.
5. Undeveloped (U) ovary from 112 mm. shrimp taken November 15, 1944, cross-section of abdominal lobes. × 14.
6. Ripe ovum, showing nucleus, yolk globules and rod-shaped peripheral bodies. YG—yolk globule; PB—peripheral body. × 279.
7. Spent (Sp.) ovary, cross-section of abdominal lobe of recently spawned shrimp. New crop of ova being produced. NO—new ovum; RO—resorptive ovum. × 35.
8. Spent (Sp.) ovary, cross-section of abdominal lobe showing almost complete recovery. Ovary is largely filled with dense masses of follicle cells and new oocytes. RO—resorptive ovum; ZP—zone of proliferation. × 52.

PLATE E









above, the oviduct is a slender though doubtless distensible tube connected with the ovary at the tip of one of the lateral lobes. No evidence was found of any branches of the oviduct extending beyond its junction with the ovary. In the ripe stage the ovary is very compactly filled with apparently no passageway through which the eggs might be directed to the mouth of the oviduct.

As the ovarian wall itself contains no muscle tissue, spawning is most likely brought about through the coordinated contracture of the cephalothoracic and abdominal muscles surrounding the ovary. It is probable that pressure is applied in such a way as to cause the eggs to move generally in the direction of the opening of the oviduct. Heldt (1938) observed the spawning of *P. trisulcatus* in an aquarium. She states that the female while resting on the bottom of the container emitted the eggs in great abundance. The process was completed in a few minutes.

As previously mentioned, a study of the "spent" ovary of *P. setiferus* reveals a large number of unspawned eggs in various stages of resorption (Plate D, 7 and 8), thus indicating that the ovary is not emptied in the spawning process and that a certain percentage of eggs never reach the exterior to be fertilized. Contrary to this, Heldt (1931) reports that in the spawning of *Penaeus caramote* Risso the ovary empties completely in one or two minutes.

The Male Reproductive System

Gross Anatomy

The male reproductive system (Plate A, 1 and Plate B, 1) includes paired testes, paired vasa deferentia and a petasma. The testes are unpigmented, translucent organs occupying a position in the body cavity very similar to that of the ovaries in the female, with the exception, however, that the testes lack the greatly extended abdominal lobes possessed by the ovaries. Each testis has projecting from its main axis an anterior lobe, apparently six lateral lobes, and a single short posterior lobe. The main trunks are more or less united for their entire lengths. In the young shrimp the testes are extremely delicate, transparent structures which can be located and removed only with great difficulty. As the animal matures, the testes change little in appearance, except to grow in size and become somewhat opaque.

The vasa deferentia arise from the posterior margins of the main axes of the testes and open to the exterior through genital pores located medially on the coxae

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Plate E

1. Undeveloped (U) ovary, cross-section of abdominal lobe. ZP—zone of proliferation. × 170.

2. Early developing (D) ovary, cross-section of abdominal lobe. ZP—zone of proliferation. × 82.

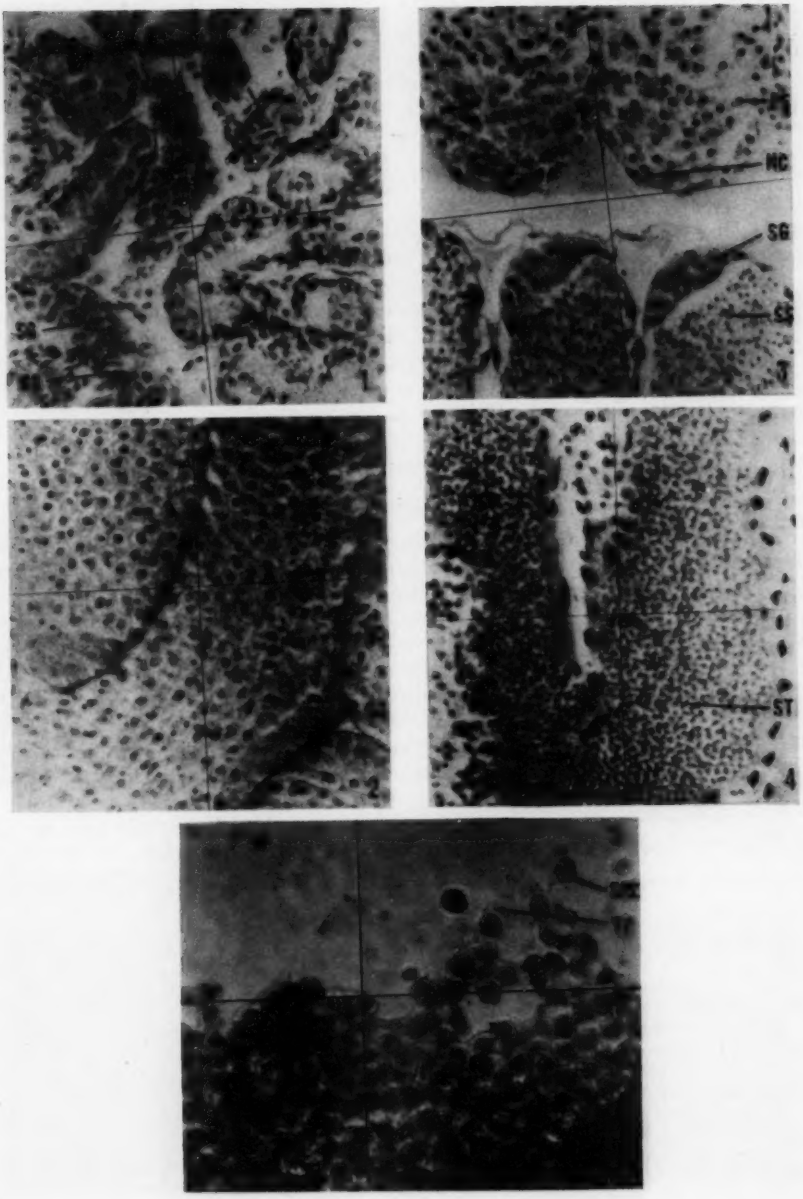
3. Advanced developing (D) ovary, cross-section of abdominal lobe. ZP—zone of proliferation; F—follicle cell. × 46.

4. Yellow (Y) ovary, cross-section of abdominal lobe; cytoplasm of ova is filled with yolk globules which take on a deep eosin stain. ZP—zone of proliferation. × 24.

5. Ripe (R) ovary, cross-section of abdominal lobe; peripheral bodies present in ova; young oocytes still being produced. ZP—zone of proliferation. × 27.

6. Oviduct, cross-section, showing thick outer wall and inner lining of tall columnar epithelium. × 102.

PLATE F



of the 5th pereiopods (Plate B, 1 and Plate C, 2). Each vas deferens consists of four distinct regions (Plate C, 1): (1) a short, narrow, proximal section passing abruptly into (2) a thickened medial portion having a double flexure which tapers to form (3) a relatively long narrow tube terminating in (4) a greatly dilated muscular region, the ductus ejaculatorius, or terminal ampoule. Here in this distal region is formed the spermatophore, the structure in which the sperm cells are conveyed from male to female.

The petasma (Plate C, 2 and 3) is a complicated membraneous structure of folds and troughs resulting from great modifications of the endopodites of the first pair of pleopods. The two symmetrical components are not fused but are held together in the mid-line by numerous, interlocking and very minute hooks, resembling a zipper in appearance and effect. The petasma supposedly functions in the transfer and attachment of the spermatophore to the ventral surface of the female.

In *P. setiferus* the endopodites of the second pair of pleopods are slightly modified to form the "appendix masculina," which may also assist to a small extent in the handling of the spermatophore.

The spermatophore is roughly pod-like in design (Plate B, 4 and 5), each terminal ampoule contributing a part, the halves being assembled outside the body immediately following their expulsion. The anterior end (as when in position on the female) of the spermatophore bears a pair of "wings" which are important in anchoring the structure in place. The posterior-dorsal region is extended to form a flange or shelf which also functions in attachment. It has been determined by dissection and by forcibly expelling the halves of the spermatophore with thumb and forefinger that each half is discharged from the terminal ampoule with the winged end foremost. And also that the right and left halves of the spermatophore, as in position on the female, are produced by the corresponding right and left terminal ampoules of the male, i.e., the right half originates in the right ampoule.

Never having observed the union of the sexes of the Penaeidae and being unable to find a description of the process in available literature, we can only attempt to imagine how this transfer must take place. It is assumed that the halves of the spermatophore are discharged almost simultaneously and with the aid of the walking legs are fitted together and placed in the clasping-like trough on the ventral surface of the petasma. In view of the origin and final orientation of the spermatophore, it appears necessary that the male and female should assume a head-to-tail position with their ventral surfaces in close proximity, in order to effect the transfer of the spermatophore. It is possible, however, that the petasma does not play as im-

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PLATE F

1. Section of immature testis containing spermatogonia and follicle cells. SG—spermatogonium; NC—nutritive cell. × 289.

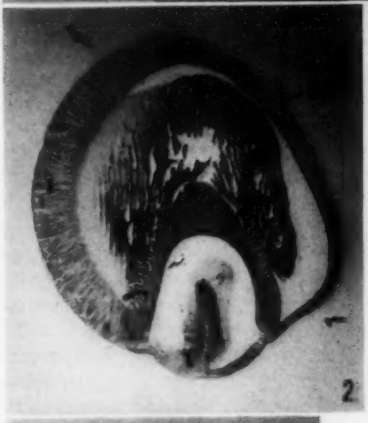
2. Section of mature testis, each tubule containing spermatogonia, primary spermatocytes and secondary spermatocytes. SG—spermatogonium; PS—primary spermatocyte; SS—secondary spermatocyte; NC—nutritive cell. × 289.

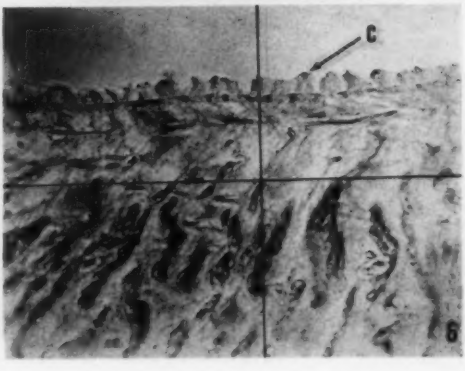
3. Section of mature testis, primary spermatocytes and secondary spermatocytes in separate but adjoining tubules. SG—spermatogonium; PS—primary spermatocyte; SS—secondary spermatocyte; F—follicle cell. × 289.

4. Section of mature testis, showing spermatids. ST—spermatid. × 289.

5. Spermatozoa from medial region of vas deferens. SPZ—spermatozoan; TP—tail piece. × 850.

PLATE G





portant a role as is generally assumed and that the walking legs may possess sufficient dexterity to manipulate the spermatophore so as to effect its attachment with the male and female in a head-to-head position.

When fixed in position on the female (Plate A, 4), the anterior end of the spermatophore is in close proximity to the openings of the genital pores. The factors coordinating the dehiscence of the spermatophore with the expulsion of the eggs are as yet unknown; however, it may be that the fluid accompanying the eggs has a chemical or physical effect causing the spermatophore to open and release the sperm at the appropriate moment.

Histology and Development

The testis

The thin wall or cortex surrounding the testis consists of two layers: an outer epithelium, and an inner layer of connective tissue. No muscle tissue is present. The body of the testis is composed of a mass of very minute, convoluted, seminiferous tubules in which the male reproductive cells are produced. It appears that the membrane-like wall of each tubule is also of two layers: the outer tunica or membrana propia, and the inner germinal epithelium.

The content of the tubules varies with the size of the shrimp and also with the season. For example, the testes of a young 130 mm. shrimp in February are much farther advanced than the testes of a 130 mm. shrimp in November. The tubules of small shrimp were found to contain spermatogonia and scattered nutritive or nurse cells (Plate F, 1). The spermatogonia have fairly definite cell walls and large round nuclei surrounded by a distinct mass of protoplasm. The nurse cells have irregularly-shaped nuclei imbedded in a syncitial mass of protoplasm with no visible cell boundaries.

As the season advances and the shrimp grows, the testes and the individual tubules increase greatly in size. Each spermatogonium passes through a period of quick growth to become a primary spermatocyte. A reduction division results in two secondary spermatocytes. These divide again to produce four spermatids which develop without further divisions into spermatozoa. The primary spermatocytes are larger than the secondary spermatocytes (Plate F, 2 and 3), which are in turn larger than the spermatids (Plate F, 4). In tubules containing these later stages, the nurse cells appear to be confined to the peripheral region of the tubule.

Once a shrimp reaches sexual maturity, the testes produce a continuous crop of sperm cells. At almost any time of the year an examination of the mature testis will reveal germ cells in all stages of development. Most of the tubules in cross-

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PLATE G

1. Vas deferens, cross-section, in proximal region. × 49.
2. Vas deferens, cross-section, in medial region, showing refractory sperm mass and extremely tall columnar epithelium lining cavities. SM—sperm mass. × 22.
3. Vas deferens, cross-section, in distal region. × 88.
4. Terminal ampoule, longitudinal-section, of immature shrimp. × 17.
5. Terminal ampoule, cross-section, of mature shrimp; spermatophore in process of formation. SP—spermatophore. × 14.
6. Cilia-bearing epithelium from terminal ampoule. C—cilia. × 367.

section show two typical stages but adjoining tubules may contain cells in different stages of development. The mature tubule (Plate F, 2) may normally contain along one margin a layer, several cells in thickness, of spermatogonia and primary spermatocytes. The main bulk of the tubule will be filled with secondary spermatocytes or spermatids or both.

The spermatozoa

Ripe spermatozoa were observed only in the vas deferens. The spermatozoan (Plate B, 3 and Plate F, 5) is composed of three typical parts: head, middle piece, and tail. The head is large and almost circular in outline, the middle piece is short and considerably more slender, while the tail is relatively thick and short. From its structure it is a logical assumption that the spermatozoan is capable of movement.

The vas deferens

The vas deferens leaves the testis as a simple tube (Plate G, 1) lined with cilia-bearing columnar epithelium and with some muscle and connective tissue in its outer wall. As the diameter of the tube increases, a longitudinal septum appears in its interior separating the tube into two channels of unequal diameter (Plate G, 2). The sperm cells move down the larger channel, their flow doubtlessly being facilitated by the beating of cilia and by secretions of the glandular epithelium. Within the smaller channel there develops from the margin a longitudinal fold which may be traced distally to the terminal ampoule. The epithelium lining the smaller channel produces a fluid which is probably involved in the formation of the spermatophore. This secretion and also the sperm mass become very hard and brittle when passed through the usual fixing and imbedding procedures, thereby making the sectioning process difficult.

After the second bend the vas deferens is reduced in diameter to form a narrow tube (Plate G, 3) which joins with the terminal ampoule. At the beginning of the narrow portion, the longitudinal septum becomes detached from the wall at one of its margins, forming a shelf or fold which continues to the terminal ampoule and parallels the other fold previously described. With the severing of this partition, the sperm-bearing fluid and the secretion of the other channel may come in partial contact for the first time. The arrangement of the folds, however, would tend to prevent any extensive intermingling of the two substances.

The greatly dilated distal region, or terminal ampoule (Plate G, 4 and 5), possesses a thick muscular wall lined with extremely tall columnar epithelial cells. From our observations the detailed composition of the wall is as follows: first a thin outer squamous epithelium underlain with connective tissue, several bands of circular muscles, then a wide zone which appears to be composed of interspersed longitudinal and circular muscle fibers, another layer of connective tissue, and finally the thick glandular epithelium. The latter contains enormous nuclei and bears cilia in certain areas. The numerous folds and partitions are formed by extensions of columnar epithelium supported by connective tissue.

In cross- or longitudinal sections the terminal ampoule of the mature shrimp presents a very complex picture. Examination of these organs from a number of individuals showed, however, that the general arrangement was quite uniform

for all. The most easily identifiable structures of the developing spermatophore are the sperm cells enclosed in a sheath and surrounded by a mass of chitinous material. In some sections the wings of the spermatophore may be seen in process of formation. Because of the refractory nature of the contents of the ampoule following fixation and imbedding, it is extremely difficult to obtain good sections for study.

Fasten (1917) has called attention to the fact that cilia have been considered universally absent from the Arthropoda. He found them, however, to occur in the vasa deferentia of Anomura and Brachyura. As reported here, striking examples of these processes are found also in the male tract of the Peneidea.

Summary

The general anatomy and histology of the reproductive organs of the shrimp, *Penaeus setiferus* (Linnaeus), have been studied and described.

Although the cytological phenomena of oogenesis and spermatogenesis have not been completely worked out, gross developmental changes in size and coloration of the organs have been related to histological and cytological changes so that the field worker, without access to a microscopy laboratory, may more accurately determine and record the stage of development.

Observations have been included on the processes of impregnation and ovulation.

Unsuccessful efforts were made to find clues which would serve as an index to the age of a shrimp. Evidence was found, however, indicating that a female may spawn more than once in a single breeding season.

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ROTATORIA FROM PENIKESE ISLAND, MASSACHUSETTS, WITH A DESCRIPTION OF PTYGURA AGASSIZI N. SP.

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This paper is based on material collected from the three main ponds on Penikese Island, Massachusetts on August 3 and 17, 1947. The collections were made in connection with the celebration of the 75th anniversary of the founding by Louis Agassiz of the Anderson School of Natural History. A large group of biologists visited the island for the purpose of making a commemorative biological survey. I am indebted to my wife who did much of the collecting and sorting of material.

Penikese Island is near the end of the chain of Elizabeth Islands leading southwest from Woods Hole, at latitude 41° 27′ N. and longitude 71° 51′ W. All three ponds were small and shallow, but Typha Pond was nearly dry. This pond was, however, nearly fresh, while the other two contained considerable concentrations of salt. While this paper is limited to aquatic species of rotifers, those living in terrestrial moss were collected by Mr. Andre Burger of Neuchâtel, Switzerland, and will be reported by him (1948).

All the species listed are widely distributed and have been reported from salt water before, with the exception of the two species of *Ptygura*.

List of Species

Tub Pond, August 3, 1947. Salinity 34 °/₀₀

Brachionus plicatilis Müller

Typha Pond, August 3, 1947. Salinity 5 °/₀₀

Brachionus calyciflorus Pallas
Cephalodella catellina (Müller)
Colurella obtusa (Gosse)
Lecane bulla (Gosse)

South Pond, August 3 and 17, 1947. Salinity 15 °/₀₀

Collotheca ornata (Ehrenberg)
Colurella colurus (Ehrenberg)
Lecane closterocerca (Schmarda)
Lecane grandis (Murray)
Ptygura agassizi n. sp.
Ptygura crystallina (Ehrenberg)

Notes on Species

Cephalodella catellina (Müller)

There has long been some uncertainty about the proper terminology of the *catellina* group of species. Since Müller's original figure cannot certainly be as-

sociated with any one of the species, it is necessary to take Ehrenberg's description as the first adequate definition, and the basic issue then becomes the identity of the form called *catellina* by Harring and Myers with Ehrenberg's species. The specimens found in Typha Pond agree well with those described as *catellina* by Edmondson and Hutchinson (1934) or *myersi* in Wiszniewski's careful study (1936).

Colurella colurus (Ehrenberg) (Fig. 11)

The few specimens collected agree fairly well with Lucks' description of *compressa* (1912), synonymized with *colurus* by Harring (1913). However, they were considerably more elongate than any *colurus* yet described. Since the genus is in need of a complete revision, no discussion is attempted here.

Lecane grandis (Murray)

The specimens in general agree well with Murray's description (1913). There seems to be some variation in the degree to which the coxal plates project beyond the ventral plate of the lorica. The lateral spines were absent from the toes of one specimen, but this character is known to be variable in other species of the genus.

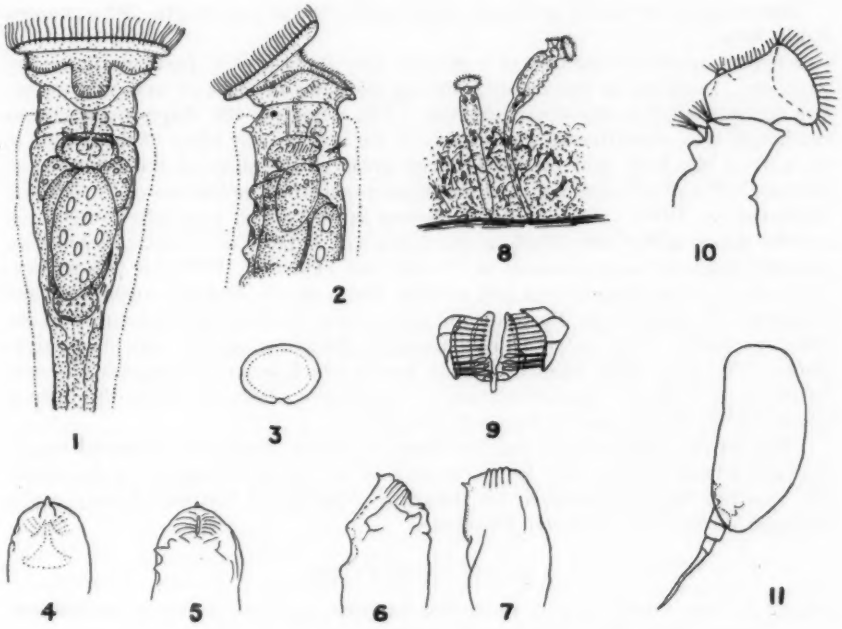
Ptygura crystallina (Ehrenberg) (Fig. 10)

One specimen was found which appeared to be a rather young *P. crystallina* (Fig. 10). The dorsal antenna was considerably more conspicuous than usual, and it is not entirely certain that this is indeed *crystallina*. The tube was fully formed and, in addition, there was a thin gelatinous layer closely adhering to the cuticle of the body. Such a covering has not been observed on specimens in fresh water.

Ptygura agassizi n. sp. (Figs. 1–9)

The corona is broadly elliptical with a very narrow dorsal gap and no ventral notch (Fig. 3). Its plane is at a fairly large angle to the axis of the body, in some positions looks almost perpendicular (Fig. 8). The buccal area is large relative to the corona, about half as long as the vertical axis. The cuticular areas lateral to the buccal area are wide and flattened, giving a characteristic appearance to the head in oblique views. The neck is about as long as deep. The body is about four-fifths as wide as the corona, fusiform, widest near the anterior end and tapering gradually to the foot. The dorsum has five or more prominent wrinkles, the most anterior of which encircles the body and separates it from the neck. The foot is heavily wrinkled and is usually about one and a half or two times as long as the rest of the body, but is very extensible and in active specimens may be three times as long. The peduncle is a short cylinder considerably narrower than the foot.

The lateral antennae are minute setate cylinders near the ventral side of the neck. On the dorsal side of the neck is a small sharply pointed spine. No setae were observed on it and it is not now possible to decide whether the spine represents a rudimentary dorsal antenna, or whether that organ is absent altogether, and this spine is comparable to that of *Ptygura melicerta* var. *mucicola*. A number of mature adults retained the eyes near the dorsal side of the neck (Fig. 2), but



EXPLANATION OF FIGURES

FIGURE 1. *Ptygura agassizi*, ventral view.
FIGURE 2. *Ptygura agassizi*, lateral view.
FIGURE 3. *Ptygura agassizi*, corona.
FIGURE 4. *Ptygura agassizi*, anterior end, fully contracted, dorsal view.
FIGURE 5. *Ptygura agassizi*, same specimen, ventral view.
FIGURE 6. *Ptygura agassizi*, same specimen, lateral view.
FIGURE 7. *Ptygura agassizi*, anterior end, partly contracted, ventral view.
FIGURE 8. *Ptygura agassizi*, two specimens showing characteristic attitude.
FIGURE 9. *Ptygura agassizi*, trophi, right manubrium partly folded down.
FIGURE 10. *Ptygura crystallina*, lateral view.
FIGURE 11. *Colurella colurus*, lateral view.

this was not true of all. The trophi are of the usual type. The first three teeth of the unci are definitely larger than the others.

The tube was difficult to observe because the Ruppia to which the animals were attached was covered with a layer of flocculent material containing bacteria and many capsulated algae. Apparently the tubes of the rotifers were short, irregularly shaped and made of rather loose gelatinous material. In addition to the usual tube, the animals were covered by a thin gelatinous layer adhering closely to the cuticle, shown by dotted lines in Figures 1 and 2. This cannot be taken as a specific character, for *P. crystallina* also had such a sheath; possibly this is an effect of salinity.

Dimensions of a typical specimen: total length, 480 μ; foot length, 285 μ; corona width, 90 μ.

Ptygura agassizi resembles in a general way *P. melicerta,* particularly variety *mucicola.* It differs in the shape of the dorsal spine and lack of any other structure corresponding to the dorsal antenna. The details of the shape of the corona and buccal area are different, particularly in the angle of the plane of the corona to the axis of the body and in the flattened areas at the sides of the buccal area. Moreover, *P. agassizi* does not have the rigorous substrate limitations of *P. melicerta* (Edmondson, 1944). One of the specimens corresponded especially closely, except for dorsal spines, with Hudson and Gosse's description of *Oecistes serpentinus,* generally regarded as a synonym of *P. melicerta* (Harring, 1913); it was particularly active in contracting foot and corona, had a poorly shaped, small tube, and contained oil globules in the posterior part of the body as did a number of the other specimens. The external morphological characters agreed with those given above. The only other members of the genus which seem to have much resemblance to this are *P. tihanyensis* and *P. stephanion,* both of which differ from *agassizi* in the corona, and lack of a dorsal spine.

This species occurred in some abundance in South Pond, and is named for the zoologist whose memory was being honored by the biological survey of the island. The type has been deposited in the American Museum of Natural History, and a paratype in the Yale Peabody Museum.

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THE EFFECT OF NITROGEN MUSTARDS ON THE RESPIRATION AND FERTILIZATION OF SEA URCHIN SPERM AND EGGS [1]

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The halogenated alkyl amines, the so-called nitrogen mustards, are extremely interesting compounds to the biologist. When dissolved in water they give quaternary nitrogen transformation products which in small amounts inhibit mitosis (Friedenwald and Schultz, 1943; Bodenstein, 1947) and produce mutations (Auerbach *et al.*, 1943; Miller and McElroy, 1948). These transformation products act as structural inhibitors for those enzymes where choline is the substrate in the enzyme-substrate complex. Furthermore, nitrogen mustards act as inhibitors of other enzymes by virtue of the great reactivity of their ethylene immonium derivatives and of their halogen groups. All these enzyme inhibitions produce inhibition of tissue respiration and of synthesis reactions (Barron, Bartlett and Miller, In press; Barron, Bartlett, Miller, Meyer and Seegmiller, In press). The effect of nitrogen mustards on cell division of fertilized sea urchin eggs was studied by Cannan and Levy (1944) and Vislocki *et al.* (1945), who found that they produced a delay in the rate of cellular division. As continuation of the work of this laboratory on the mechanism of action of nitrogen mustards, the effect of these compounds on the respiration and fertilization of sea urchin eggs and sperm was studied. Nitrogen mustards were found to produce in sea urchin sperm a striking stimulation of respiration accompanied by an inhibition on fertilization of eggs of the normal sequences of cell division and development.

Experimental

Male and female species of *Arbacia punctulata* were allowed to shed their sperm and eggs. The eggs were washed in a large amount of filtered sea water and the excess water was withdrawn previous to the experiments. The sperm were suspended in twelve volumes of filtered sea water, shaken, centrifuged for five minutes, and resuspended in the desired amount of sea water. The nitrogen mustards (their HCl salts) were dissolved in sea water just before use and were added to the sperm suspension 15 to 30 minutes previous to the measurement of respiration. The manometric experiments were performed at 25° C.; the fertilization experiments, at the room temperature of the laboratory, 23°–25° C.

Effect of nitrogen mustards on the respiration of sperm and of eggs of sea urchin. Freshly dissolved methyl bis (β-chloroethyl) amine HCl (MBA) at a concentration of 0.001 *M* added to sperm suspensions from 15 to 30 minutes be-

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TABLE I

Effect of methyl bis (β-chloroethyl) amine HCl (MBA) on the respiration of sea urchin sperm
The cells were suspended in sea water. Temperature 25° C.

Experiment No.	O_2 Uptake per Hour		
	Control	MBA	Increase
	c. mm.	c. mm.	Per cent
I	46.6	128.8	176
II	56.5	139	146
III	80	115	44
IV	41	84	105

fore measurement of the O_2 uptake increased it from 44 to 176 per cent (Table I). This variation in the increase of respiration is probably due to the different periods of times which elapsed from addition of MBA to the measurement of the O_2 uptake. In fact, when MBA was added while respiration was being measured, there was an induction period of about 30 minutes when the O_2 uptake continued at the same rate as before addition of MBA, the increase starting afterwards (Fig. 1). This induction period must be due to the slow formation in sea water of the active ethylene immonium transformation product (transformation of nitrogen mustards into the ethylene immonium derivative is greatly retarded in the presence of KCl and NaCl).

Increase in respiration was also shown on addition of other halogenated alkyl amines. In experiments performed under similar conditions, MBA increased the respiration of sea urchin sperm by 196 per cent; isopropyl bis (β-chloroethyl) amine HCl, 115 per cent; ethyl bis (β-chloroethyl) amine HCl, 155 per cent; and tris (β-chloroethyl) amine HCl, 79 per cent (Table II).

The increase in respiration was obtained with concentrations varying between 0.001 *M* and 0.0001 *M*. When the concentration of MBA was diminished to 0.00001 *M* there was no effect at all with sperm suspensions of 1:20. With concentrations of MBA of 0.0001 *M*, the increase in respiration, which did not start until one hour and a half after addition, remained constant for the duration of the experiment, eight hours. However, when the concentration was increased ten

TABLE II

The effect of nitrogen mustards on the respiration of sea urchin sperm

The nitrogen mustards (0.0001 *M*) were dissolved in sea water and added 20 minutes before measurement of the O_2 uptake.

Nitrogen mustard	O_2 uptake	
	Control	N Mustard
	c. mm.	c. mm.
Methyl bis (β-chloroethyl) amine HCl	42	124
Isopropyl bis (β-chloroethyl) amine HCl	42	90
Ethyl bis (β-chloroethyl) amine HCl	42	107
Tris (β-chloroethyl) amine HCl	42	75

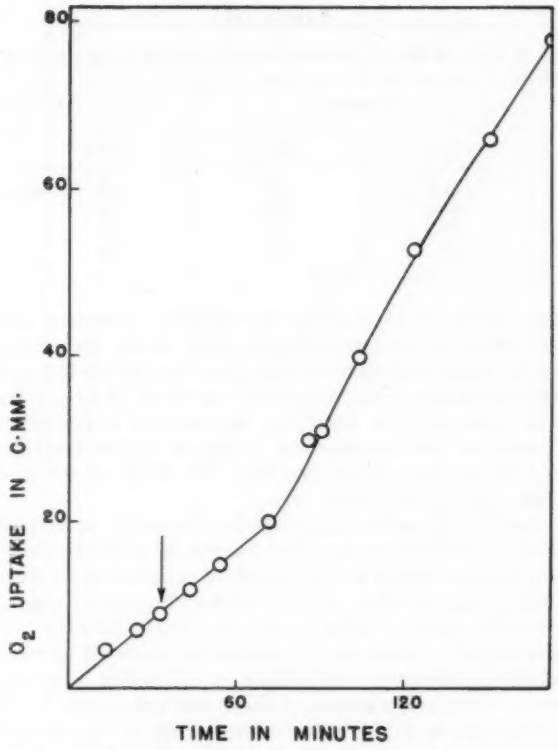

FIGURE 1. Effect of methyl bis (β-chloroethyl) amine HCl (0.001 *M*) on the respiration of sea urchin sperm. MBA was added at time marked by arrow. Temperature 25° C.

times (0.001 *M*) the increase in respiration diminished after three hours (Fig. 2). This increase of respiration followed by a diminution seemed to indicate that increase and inhibition could be obtained by either increasing the concentration of nitrogen mustard or by decreasing the concentration of sperm cells. The latter method was chosen to test this possibility. Sperm suspensions were diluted with sea water as follows: 1 of sperm and 10 of sea water; 1:50; 1:100 and 1:200. After addition of 0.001 *M* MBA, the O_2 uptake of the sperm suspensions was measured for nine hours. The most striking results were obtained on sperm suspensions diluted 1:200. In the first hour, MBA increased the respiration by 120 per cent; in the second hour this increase dropped to 54 per cent; and in the third hour it disappeared completely. In the fourth hour the inhibition started (27 per cent), to become complete in the sixth hour (Fig. 3). Sperm suspensions at this dilution had a remarkably high respiration, for the Q_{O_2} of the control samples was 26.0; furthermore, the O_2 uptake remained at steady values throughout the duration of the experiments, nine hours.

TABLE III

Effect of MBA (0.001 M) on the respiratory quotient of sea urchin sperm

Experiment No.	O_2 uptake		CO_2 production	
	Control	MBA	Control	MBA
I	54.5	171	50	147.5
II	62.0	164	70	140
III	76	137	75	128
IV	49	147	50	144

This increase seems to be confined to oxidative processes because nitrogen mustards had no effect on the anaerobic glycolysis of sea urchin sperm.

The increase in respiration seems to take place on the whole respiratory process as shown by measurements of the respiratory quotient (R.Q.). The R.Q. of sea urchin sperm is close to 1 as found by Barron and Goldinger (1941). The average R.Q. value of four experiments (triplicate determinations for each experiment) was 1.01 for the control samples. The R.Q. of the nitrogen mustard containing samples was 0.93 (Table III).

The respiration of sea urchin eggs whether fertilized or unfertilized was not affected by addition of MBA or isopropyl bis (β-chloroethyl) amine HCl (Table IV). This lack of effect cannot be due to lack of penetration for nitrogen mustards added to sea urchin eggs produce a delay in the cleavage of eggs when they are fertilized with normal sperm (Cannan and Levy, 1944; Vislocki *et al.*, 1945).

Inhibitions of enzymes and of tissue respiration produced by nitrogen mustards can be prevented on addition of thiosulfate or of choline (Barron, Bartlett and Miller, In press). Previous addition of these two substances to sperm at a concentration 50 times that of MBA had no effect at all on the increased respiration produced by the nitrogen mustard. The increase in respiration was not associated with any possible combination of seminal fluid with the nitrogen mustard (if we accept the postulated inhibition of respiration by seminal fluid), for stimulation of

TABLE IV

Effect of MBA and isopropyl bis (β-chloroethyl) amine HCl on the respiration of sea urchin eggs

Nitrogen mustard (0.001 *M*)	O_2 uptake in 1 hour			
	Unfertilized eggs		Fertilized eggs	
	Control	N. mustard	Control	N. mustard
MBA	19.1	16.8	40	37
MBA	17.0	15.0	46	44
Isopropyl bis (β-chloroethyl) amine HCl	20	18	52	54
Isopropyl bis (β-chloroethyl) amine HCl	21	23	32.5	31.5

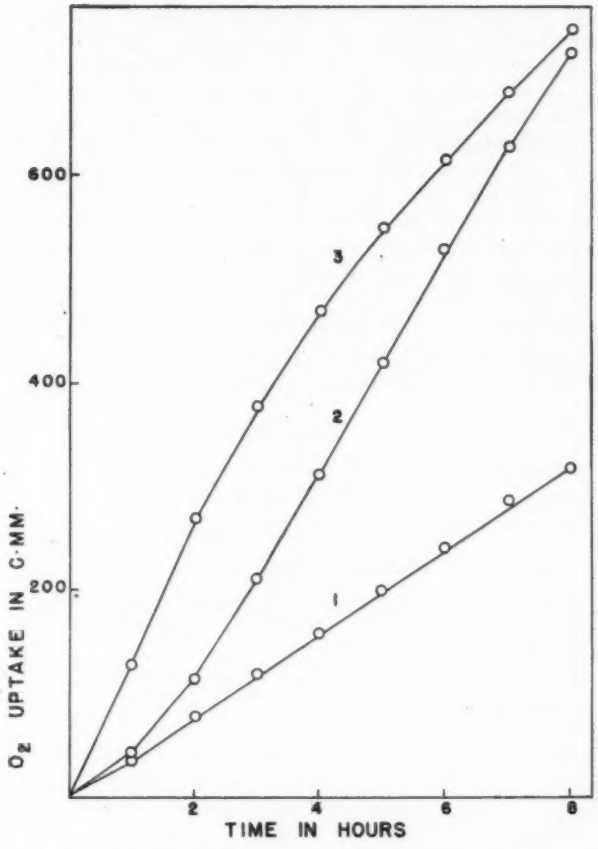

FIGURE 2. Increase in respiration produced by MBA on sea urchin sperm. Effect of concentration of MBA. 1. Control; 2. MBA, 1×10^{-4} *M*; 3. MBA, 1×10^{-3} *M*.

respiration was obtained on sperm suspensions washed repeatedly with sea water by successive suspensions and centrifugation.

Effect of MBA on the fertilization of Arbacia eggs. Cannan and Levy (1944) and Vislocki *et al.* (1945) found that nitrogen mustards in small concentrations produced a delay in the rate of cleavage of sea urchin eggs. In their experiments 0.0013 *M* MBA was added to a suspension of eggs, and 20 minutes later the eggs were washed and fertilized with non-treated sperm. Under those conditions, while first cleavage (50 per cent) took place in 56 minutes in the control eggs, it required 78 minutes in the MBA treated eggs. Treatment just before fertilization started retarded first division but not the second division. The inhibition of cell division was found more effective when the sperm was treated with nitrogen mustard, as can be seen in the two series of experiments.

First series of experiments. A suspension of sperm (1:50) was added to different concentrations of MBA to give the following concentrations: 1×10^{-3} *M*; 1×10^{-4} *M*; 1×10^{-5} *M*; and 1×10^{-6} *M*. Forty minutes later, 0.1 cc. of this sperm suspension was added to 20 cc. of sea water containing unfertilized eggs. During the first cleavage period the eggs receiving sperm suspensions with an initial MBA concentration of 1×10^{-3} *M* and 1×10^{-4} *M* (concentration in the egg suspension, 5×10^{-5} and 5×10^{-6}) showed only 1 and 3 per cent of eggs in the two-cell stage. The eggs receiving sperm with an initial MBA concentration of 1×10^{-5} *M* and 1×10^{-6} *M* showed 75 per cent and 92 per cent cells in the two-cell stage. Thirty hours later, when the control cells were all motile, in the blastula and pluteus stages, the cells receiving sperm with MBA at the initial concentrations of 1×10^{-3} *M* and 1×10^{-4} *M* were all nonmotile; the cells receiving sperm with MBA concentration of 1×10^{-5} *M* had 30 per cent of motile cells and those receiving sperm with 1×10^{-6} *M* MBA had 50 per cent of motile cells. Furthermore a number of morphological alterations were observed (Table V).

TABLE V

Effect of MBA added to sperm on the fertilization and development of sea urchin eggs
The sperm was diluted 1:200.

Time after fertilization	Control	Initial concentration of MBA, *M*			
		1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
Minutes	Per cent	Per cent	Per cent	Per cent	Per cent
66—First cleavage 2 cell stage	80	1	3	75	92
118—Second cleavage 2 cell stage	18	65	85	20	18
4 cell stage	75	None	3	74	82
180—2 cell stage	—	4	2	2	
4 cell stage	10	39	7	3	2
Many cell stage	90	37	87	95	98
25 hours—Blastula Motile	60	3	20	75	60
30½ hours—Motile Blastula	All	None	None	30	50

Second series of experiments. Suspensions of sperm were treated with 1×10^{-3} *M* MBA and twenty minutes later 0.05 cc. of this suspension was added to a suspension of eggs in 5 cc. sea water. A suspension of unfertilized eggs was treated with an amount of MBA corresponding to that received by the eggs fertilized with MBA treated sperm; twenty minutes later they received normal sperm. At the end of 158 minutes, while the control and the treated eggs had 90 per cent of cells in the 4-cell stage, the eggs receiving treated sperm had only 39 per cent. Twenty-seven hours later the control and the MBA treated eggs had the same amount of

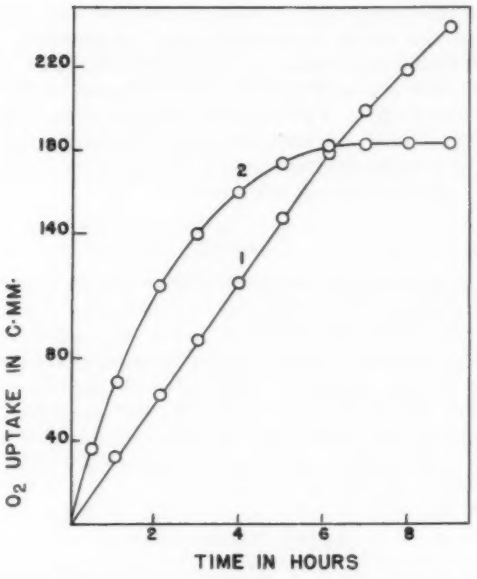

FIGURE 3. The effect of MBA (0.001 *M*) on the respiration of dilute sperm suspensions. Dilution, 1:200. $Q_{O_2} = 26.0$; 1. Control; 2. MBA.

actively moving pluteus (45 per cent) while the eggs receiving treated sperm had only 8 per cent of motile pluteus and had 16 per cent of cells morphologically abnormal.

Delay in the rate of cell cleavage, decrease in the number of cells reaching the pluteus stage, as well as abnormal cells, were found even when sperm and MBA were in contact for only two minutes before fertilization.

DISCUSSION

The sperm of sea urchin is a striking exception in its response to the addition of nitrogen mustards. While other cells or tissues are either inhibited or not affected in their respiration, the sperm of sea urchin showed a marked increase. This increase, which comprises the whole respiration process there being no alteration in the respiratory quotient, must be due to the action of nitrogen mustards on the regulatory mechanisms of the cell, i.e., those mechanisms which control the equilibrium relationships between anabolic and catabolic processes. The increase in respiration seems to occur, however, only when small concentrations of nitrogen mustard penetrate into the cells, for when the cell concentration was decreased complete inhibition of respiration followed the initial stimulation. Undoubtedly, as more nitrogen mustard penetrated the cells a concentration was reached—enough to produce the known enzyme inhibitions found by Cori (1943), Dixon (1942) and Barron *et al.* (In press). The stimulation of respiration might be due to

combination of the halogen groups of nitrogen mustard with soluble – SH groups in the sperm cell, groups which act as regulators of respiration. Nitrogen mustard produced also a profound impairment in the fertilization power of sperm. In fact, sperm treated with nitrogen mustard was more effective in inhibiting the rate of cell division of fertilized eggs. Furthermore, the eggs were stopped in their development so that none reached the pluteus stage. The mechanism of this inhibition is not yet known; it is undoubtedly related to the property of nitrogen mustards of inhibiting mitosis and of producing mutations.

Summary

The halogenated alkyl amines, methyl bis (β-chloroethyl) amine HCl, isopropyl bis (β-chloroethyl) amine HCl, ethyl bis (β-chloroethyl) amine HCl, and tris (β-chloroethyl) amine HCl, when added to sperm suspensions of *Arabacia punctulata* increased their respiration. This increase seems to affect the entire respiratory process as shown by the unaltered R.Q. value. Anaerobic glycolysis was not altered. With dilute sperm suspensions (1:200) the stimulation of respiration was followed by inhibition. Sperm so treated produced, on fertilization of eggs, an inhibition in the rate of cleavage, of pluteus formation, and motility of blastula, and produced in addition a number of malformations.

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A STUDY OF THE CHROMOSOMES IN TWO SPECIES OF BATS (CHIROPTERA)[1]

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The Chiroptera is an order of mammals in which the chromosome survey has progressed less than in other orders and, despite their wide distribution all over the world, our cytological knowledge of them has remained incomplete. With reference to the evolution of mammals, the order Chiroptera occupies a very important position, since they are closely related to the Insectivora, which in turn are of interest in their relation to the Primates. In view of these facts a thorough exploration of the chromosomes of these mammals is highly desirable.

The literature pertaining to the chiropteran chromosomes is filled with discrepancies. Van der Stricht (1910), the first to concern himself with the chiropteran chromosomes, recorded the approximate number as 9–10 in the oocytes of *Vesperugo noctula*. Athias (1912) in an incomplete account of the chromosomes of *Rhinolophus hiposideros* and *Vesperugo serotinus* reported about 16 chromosomes as haploid in the oocytes of the former species and 15–24 in the latter. Jordan (1912) counted over 24 chromosomes in the diploid group of a male bat (the species name not given) and reported an XO chromosome mechanism. Working with a species of bat (the name unknown), Hance (1917) reported an approximate number of 40 for the male diploid complex. One is impressed from the above accounts that these earlier workers were handicapped by inadequate technique and any conclusive evidence cannot be expected from them. The only author who succeeded to some extent in surveying the morphology of chromosomes in this field is Painter (1925). He determined the diploid number of chromosomes in the embryonal cells of the horse bat, *Nyctinomus mexicanus*, to be 48, but presented no evidence relating to the maturation divisions.

The present paper deals with the chromosomes of the Okinawa fruit bat, *Pteropus dasymallus inopinatus* Kuroda, a member of the Megachiroptera, and those of the Japanese horse-shoe bat, *Rhinolophus ferrum-quinum nippon* Temminck, belonging to the Microchiroptera. In the former species, the material proved favorable for a thorough study of chromosomes throughout spermatogenesis, while in the latter form only the primary spermatocytes came under observation because of the unfavorable season for collecting the material. But in view of the unsatisfactory status of the chromosome survey in this order, the publication of even such a fragmentary account seems justified.

It is pleasant to express the author's sincere appreciation to Dr. Kan Oguma for valuable suggestions. Cordial thanks are also due to Professor Franz Schrader of Columbia University for his kind service in revising the manuscript and in its publi-

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cation. Gratitude is extended to Dr. Y. Yamashina and the late Dr. D. Nakamura through whose generosity the material for this study was made available. Financial aid from the 4th Special Committee for Researches of Genetics in the Nippon Gakuzyutsu-Shinkokwai, as well as from the Scientific Research Expenditure of the Department of Education is also acknowledged here.

The chromosomes of Pteropus dasymallus inopinatus Kuroda

Pteropus dasymallus inopinatus Kuroda, commonly known as the Okinawa fruit bat, is a member of the Pteropodidae of the Megachiroptera. It is distributed throughout the Riu-Kiu Islands. Testicular material obtained from two specimens caught in different localities was employed for study. One of them came to hand through the generosity of Dr. Y. Yamashina and was killed in September 1940. This animal came from Okinawa and had been reared for a few years in the Yamashina Ornithological Institute, Tokyo. The other specimen was secured by the author at Nago, Okinawa in April 1941. The testes were removed in living condition and fixed in Flemming's solution with no trace of acetic acid. Subjected to the usual paraffin method, the sections were stained with Heidenhain's iron-haematoxylin and light-green. The observations were chiefly carried out on the former material, which was fixed at a more favorable season of the year.

Careful counting of several adequate metaphase figures of the spermatogonia gave 38 as the diploid number in every case. As shown in Figures 1 and 2, the chromosome complement of this form is remarkable not only for its comparatively low number, but also for the fact that the majority of the chromosomes are J- or V-shaped. Close examination reveals that, except for a pair of elongated rod-shaped elements together with a Y chromosome of minute size, all the other elements are provided with atelomitic fibre attachments. The latter are submedian or subterminal in location. The autosomes vary in size, ranging from enormous J- or V-shaped elements to small, slightly curved bodies. In the equatorial plate, the latter lie with the bend directed towards the centre of the plate and with their long axes stretching across the radius of the equatorial plate.

The two elongated chromosomes, in turn, are in striking contrast to the others due to their remarkable rod-shape. They form a homologous pair, identical in length, and seem to be of telomitic nature, since they have tapering ends at their inner extremities where the spindle fibres attach. They correspond in length to one arm of the largest V-shaped chromosome.

The even number of chromosomes herein established naturally suggests the occurrence of an XY-pair of sex chromosomes, as is the general rule in the mammals so far reported. On account of its size and shape, the Y chromosome is readily distinguishable from the others. The identification of the X element, however, presents some difficulty. It is probable that the X is represented by one of the atelomitic chromosomes. To judge from the morphology of the sex chromosome pair as disclosed in the first meiotic division, the X element seems to be represented by one of the submedian chromosomes, fairly large in size and having extremely dissimilar arms.

The chromosomes found in the primary spermatocyte metaphase are observable with extreme clearness due to the reduced number of elements. The chromosome count at this stage is therefore made with certainty and thus it gives final confirma-

tion of the diploid number obtained in the spermatogonia. Every metaphase plate under observation shows consistently 19 chromosomes, having various shapes and sizes (Figs. 3 to 5). The chromosomes are all bivalent in structure. The haploid complex consists of 18 autosomal bivalents with ordinary structure and an XY complex of heteromorphic nature, composed of the large J-shaped X and the small Y.[2] Corresponding to the morphology of the spermatogonial chromosomes, the autosomal bivalents are variable in size and the majority of them appear as atelomitic in their fibre attachment. Three or four of these bivalents are relatively small in size, in striking contrast to the remaining ones. Among the larger bivalents the size differences are slight and they form a closely graded series.

The XY-bivalent is readily distinguishable from the autosomal bivalents because of its unusual asymmetrical configuration, and by its relatively peripheral position in the metaphase plate (XY in Figs. 3 to 5). It consists of the J-shaped X element of large size and the very minute Y element. The latter is attached to the extremity of the long arm of the X. At metaphase, the X is placed so that its longer arm with the Y at its extremity is vertical to the equatorial plate, while the shorter arm lies in most cases parallel to the equatorial plate. Sometimes the longer arm of the X acquires two constrictions due probably to the elongation, giving as a result a somewhat undulating appearance (Figs. 6 and 7). To judge from the structural configuration, the spindle fibres of the X attach to the point where the long and short arms join, while in the Y they apparently attach to its free end. The whole configuration displayed by the XY bivalent, as outlined in the above description, is clearly displayed in the profile view of the metaphase plate of the primary spermatocyte, as given in Figures 6 to 8.

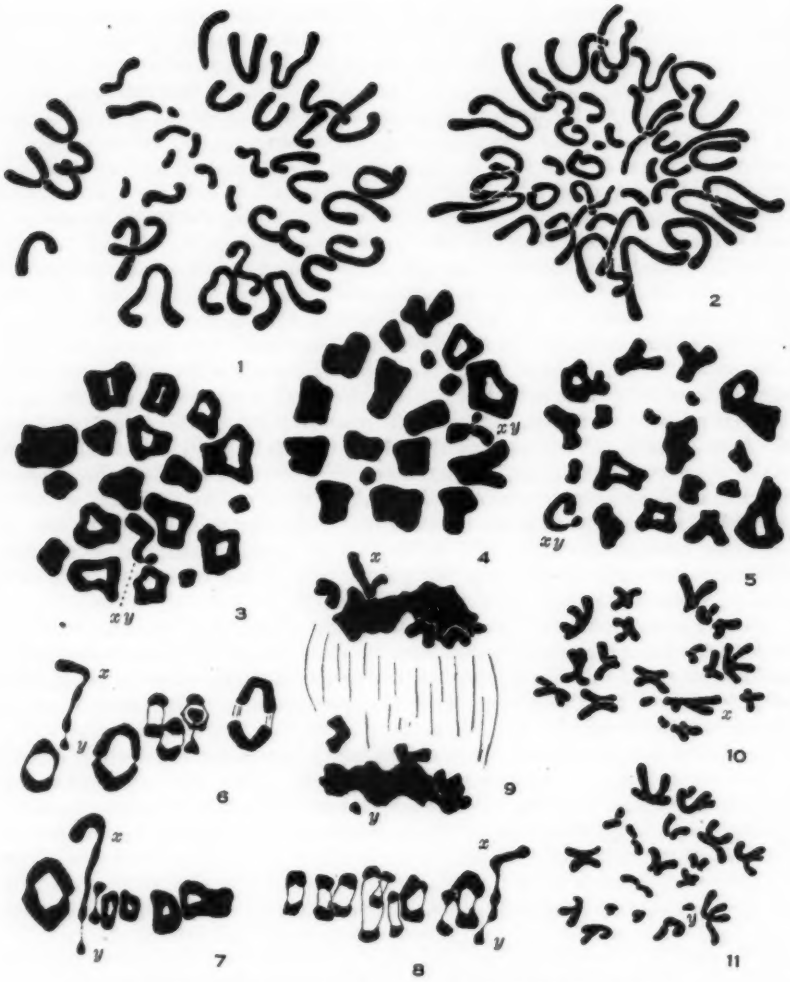
At the commencement of anaphase of the first division, the separation of autosomes takes place synchronously and there is usually no element which lags or precedes the others (Fig. 8). During the division of the autosomal elements, the X also disjoins from the Y and they migrate to opposite poles. The anaphasic picture is clearly seen in Figure 9.

There are discernible two kinds of secondary spermatocytes, though each has an equal number of chromosomes, resulting from the reductional segregation of the X and Y in the first division. The one consists of 18 autosomal elements and the X chromosome (Fig. 10), and the other contains a corresponding set of autosomal elements and the minute Y (Fig. 11).

The chromosomes of Rhinolophus ferrum-equinum nippon Temminck

The Japanese horse-shoe bat, *Rhinolophus ferrum-equinum nippon* Temminck, is a common species of the Rhinolophidae (the Microchiroptera) and known throughout Hokkaido, Hondo, Shikohu and Kyusyu. The testes of a male specimen which was obtained at Nagasaki in October 1937 were fixed with Flemming's solution without acetic acid by the late Dr. D. Nakamura. After imbedding in paraffin the material was sent to the author. Staining was done with Heidenhain's iron-haematoxylin and light-green.

[2] It will be understood that since no female material was available, the specific identification of the X and Y chromosomes is tentative. However, in view of the general agreement in the configuration of the sex chromosomes of other mammals, there would appear to be small doubt about its correctness.

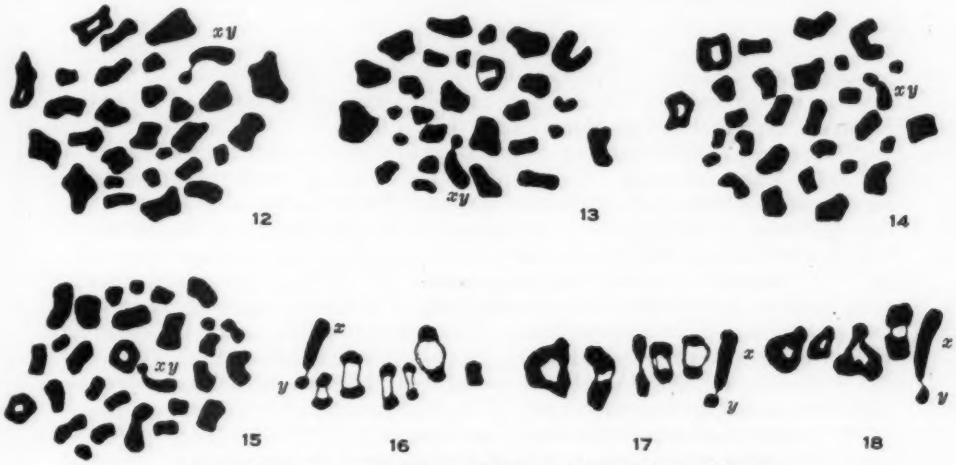


All drawings at a magnification of 3200 × (reduced from 6000 ×)

FIGURES 1–11. Chromosomes of *Pteropus dasymallus inopinatus*. 1–2, Spermatogonial metaphases, showing 38 chromosomes. 3–5, primary spermatocyte metaphases, showing 19 bivalents. 6–8, side views of the first division metaphases, showing the XY complex. 9, side view of the first division anaphase, showing the segregation of the X and Y elements. 10, secondary spermatocyte metaphase, showing 19 dyads. X-class. 11, the same, Y-class.

The material proved to be insufficient for a complete study of the spermatogenesis, because of the unfavorable season of collection; consequently only fragmentary notes concerning the primary spermatocyte chromosomes can be given here.

The haploid number of chromosomes was determined in the primary spermatocyte. The author was surprised at finding a higher number of chromosomes in this species than that encountered in the previous form. Counts in several adequate plates established that there are 29 distinct chromosomes having bivalent nature. As seen in Figures 12 to 15, the chromosomes vary considerably in their size and shape. The configuration of some of the larger bivalents is strongly suggestive of subterminal or submedian fibre attachments. To the author's view, a few of the medium sized ones seem also to be of atelomitic nature. Four or five bivalents are extremely small in size. Outstanding in the haploid complement is a heteromorphic bivalent which is composed of a long rod-shaped element and a minute granular one, connected end to end with each other. Taking all characteristic conditions into con-



All drawings at a magnification of 3200 × (reduced from 6000 ×)

FIGURES 12–18. Chromosomes of *Rhinolophus ferrum-equinum nippon*. 12–15, primary spermatocyte metaphases, showing 29 bivalents. 16–18, side views of the first division metaphases, showing the XY complex.

sideration, it is concluded that this particular chromosome configuration is the XY-bivalent, the larger component being the X and the smaller the Y (XY in Figs. 12 to 15). The XY-bivalent usually takes a peripheral position in the metaphase arrangement.

It is in the side view of the metaphase spindle that the configuration and structure of the XY-bivalent are most clearly observed (Figs. 16 to 18). Morphologically considered, the X seems to be provided with a telomitic fibre attachment. The Y element is more or less spherical in outline and seems to be slightly smaller than the smallest autosome. The two chromosomes are connected to each other, end to end, by a fine fibre and are placed nearly perpendicularly to the equatorial plate in a linear configuration. The mode of conjugation as seen in the X and the Y is fairly comparable to that found in the mice as reported by the author (Makino, 1941).

On the basis of the above results, the following statement is possible: the diploid number of chromosomes in this species would appear to be 58, comprising both atelomitic and telomitic elements of varying size and shape. The sex chromosomes are of the usual XY type, the X having an elongated rod-shape and the Y a minute dot-like one.

Remarks

It seems to the author that among the earlier works on chiropteran chromosomes the only account sufficiently accurate to warrant a more detailed discussion is that of Painter (1925) on the house bat, *Nyctinomus mexicanus*, a species of the Molossidae. Painter (1925) made the chromosome count in the amnion of young embryos and established 48 chromosomes as the diploid number of this species. Of these, at least six pairs of chromosomes have a distinct V-shape (Painter, 1925, Plate I). In comparison with the present material, it is thus evident that the number of chromosomes of *Nyctinomus* is smaller than that of *Rhinolophus ferrum-equinum nippon*, in which 29 haploid chromosomes were clearly counted. However, no precise comparison is possible with the data at hand.

The present communication constitutes the first report on the chromosomes of one of the Macrochiroptera. As reported in the foregoing pages, the diploid number of chromosomes of the Okinawa fruit bat, *Pteropus dasymallus inopinatus*, is 38. Referring to the literature on mammalian chromosomes (see the list newly compiled by Makino, 1947), it is apparent that the chromosome number here established for this species is rather low in comparison with that of other eutherian mammals, so far as the recorded cases are concerned. Furthermore, specially remarkable is the fact that the karyotype of this form is probably archaic among the eutherian species so far studied, since, excepting one pair of long rod-shaped autosomes and the Y chromosome, the chromosomes are all provided with an atelomitic fibre attachment, the majority of them being V- or J-shaped and of large size.

No accurate and clear-cut evidence has been demonstrated for the sex chromosome mechanism of the Chiroptera in any of the previous investigations. Painter (1925) working with *Nyctinomus*, was unable to observe the sex chromosome of this species in the maturation division. He stated that the constancy of the chromosome number in different embryos together with the fact that in some embryos a pair of unequal sized elements is observed, would seem to indicate that we have in this form the XY type of sex chromosome. The evidence presented in the present study shows that the XY mechanism of sex determination occurs in both of the species under study. In both the X and the Y separate in the first division, and pass to opposite poles.

Painter (1925) counted the chromosome number in 10 species of eutherian mammals covering 7 different orders, and emphasized that 48 chromosomes occur in the most primitive eutherian order of Insectivora, and in such divergent orders as the Primates and Chiroptera. He deduced from this evidence that the chromosome number of 48 seems to be the typical and is probably the basal number for the mammals.

During recent years, the present author has engaged in a comparative study of mammalian chromosomes, endeavoring to make as broad a survey of Eutheria as possible. At present his investigations have extended to 42 species which repre-

sent 1 species of the Insectivora, 2 species of the Chiroptera, 22 species of the Rodentia, 4 species of the Carnivora, 8 species of the Artiodactyla, 2 species of the Perissodactyla, 1 species of the Cetacea and 2 species of the Primates. Some of the results have already been reported (Makino, 1942, 1943 a, b, c, 1944 a, b, c, 1946, 1947 a, b). The results of these studies demonstrate that there is a striking diversity in the number of chromosomes of the species studied, ranging from 30 in the Vole, *Microtus kikuchi* to 78 in the dog, *Canis familiaris*, and further that the chromosome number of 48 was proved to exist in only four out of 42 species studied—to wit—*Buballus buffelus* of the Artiodactyla, and *Apodemus agrarius*, *A. semotus* and *Lepus gichiganus ainu* of the Rodentia. Thus the newly obtained data clearly point to the fact that the occurrence of 48 chromosomes is relatively rare in mammals, and can no longer be regarded as the basal number. It is evident that our knowledge of the chromosomes of mammals is still too scanty to justify any generalization. To discover the basal number and to establish a phylogenetical relationship on the basis of cytology is a task which will require the accumulation of accurate data in an immense number of closely related forms, before one can attempt to formulate any rule of general application.

Summary

The chromosomes of *Pteropus dasymallus inopinatus*, a species of the Macrochiroptera, were investigated in male germ cells. This species was found to possess the diploid number of 38 in spermatogonia and the haploid number of 19 in both of the primary and secondary spermatocytes. The karyotype of this form is noticeable in having an archaic constitution in comparison with that of other forms of mammals. Excepting a pair of elongated rod-shaped elements and a very minute Y chromosome, the remaining members of the diploid complex are all provided with atelomitic fibre attachment; the majority are J- or V-shaped. The sex chromosome mechanism was shown to be of the usual XY type. The X is represented by one of the large J-shaped elements, consisting of two arms of very unequal length, while the Y is a minute dot, the smallest of all. At meiosis the Y is found conjugated with the X at the free end of its longer arm. In the first division the X and the Y pass to opposite poles. There result two kinds of secondary spermatocytes, one with an X and the other with the Y.

Observations upon the primary spermatocyte chromosomes reveal that *Rhinolophus ferrum-equinum nippon* possesses a haploid number of 29. From this haploid number it may be assumed that the diploid number of this species is 58. Judging from the configuration of the bivalents, this complement comprises both telomitic and atelomitic members. A heteromorphic XY bivalent is very distinct in the haploid group. It consists of a larger rod-shaped component, the X, and a minute dot-like one, the Y, connected end to end. They segregate in the first division.

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